GLUCOSE METABOLISM IS ESSENTIAL FOR PLATELET FUNCTION

by

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ABSTRACT

Patients with diabetes display increased thrombosis and platelet activation. Preliminary metabolomics analysis of platelets from patients with T2DM revealed an accumulation of glycolytic and TCA intermediates relative to healthy controls. In vitro studies of platelets under hyperglycemic conditions suggest that glucose metabolism may lead to increased platelet activation. Platelets import glucose via two glucose transporters: GLUT1, which is expressed on the plasma membrane, and GLUT3, of which 15% resides on the plasma membrane and the remaining 85% resides on α-granule membranes. Following stimulation, platelet α-granules translocate to the plasma membrane and release their cargo. To better understand the role of glucose metabolism on platelet function we generated platelet-specific knockouts of GLUT1 (GLUT1 KO), GLUT3 (GLUT3 KO) and GLUT1+GLUT3 (DKO) using a Pf4 Cre recombinase transgenic mouse crossed to mice that harbor floxed GLUT3 or GLUT1 alleles. Generation of these mouse models allowed us to test the hypothesis that glucose metabolism is essential for platelet function.

We found that GLUT3 not only facilitates platelet activation-mediated glucose uptake, but it also facilitates glucose uptake into α-granules under basal conditions. These data indicate that GLUT3-mediated α-granule glucose uptake is essential for platelet activation, degranulation, and deletion of GLUT3 in platelets leads to increased survival in a collagen/epinephrine-induced pulmonary embolism model.

Deletion of both GLUT1 and GLUT3 (DKO) in platelets results in the complete inhibition of glucose uptake and glycolysis. DKO mice displayed thrombocytopenia (~33% reduction), decreased circulating platelet $t_{1/2}$ and had an impaired ability to increase platelet

production following treatment with antiserum. Additionally, DKO platelets had a significantly reduced ability to activate and mobilize calcium in response to multiple agonists, release α-granules, and demonstrated impaired activation of thrombosis in vivo. Together these data indicate that glucose metabolism is essential for platelet production, maintenance, activation, and in vivo thrombosis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OFABBREVIATIONS

12-HETE	12-Hydroxyeicosatetraenoic acid
12-HpETE	12-Hyroperoxyeicosatetraenoic acid
15-HETE	15-Hydroxyeicosatetraenoic acid
2-DOG	2-Deoxy-D-Glucose
AA	Arachidonic acid
ADP	Adenosine diphosphate
AEC	Adenolate energy charge
AKT	Protein kinase B
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
COX-1	Cyclooxygenase-1
FcRγ	Fc receptor Y
GLUT1	Glucose Transporter 1
GLUT3	Glucose Transporter 3
GP1bα	Glycoprotein 1bα
GP1bβ	Glycoprotein 1bβ
GPCR	G-Protein coupled receptor
GPIIbIIIa	Glycoprotien IIb/IIIa
GPVI	Glycoprotein VI
GSH	Glutathione
GSK	Glycogen synthase kinase

HEPA	13-hydroxy-14,15-epoxy-5,8,11-eicosatetraenoic acid
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
ITAM	Immunoreceptor tyrosine-based activation motif
LOX12	Lipoxygenase-12
LOX5	Lipoxygenase-5
MCAO	Middle cerebral arterial occlusion
NAC	N-acteyl cysteine
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PAR1-4	Protease Activated Receptor
PDGF	Platelet Derived Growth Factor
PF4	Platelet Factor 4
PI3K	Phosphoinosidide 3-kinase
PMCA	Plasma membrane calcium ATPase
PPP	Pentose Phosphate Pathway
RA	Rheumatoid Arthritis
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted protein
ROS	Reactive oxygen species
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SFK	Src Family Kinase
SLC2	Solute carrier 2
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus

TGF	Transforming Growth Factor
tPA	tissue plasminogen activator
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

Platelets are small anuclear cells generated by megakaryocytes that circulate in the blood for 6 to 10 days in humans (4 to 6 days in mice) and maintain hemostasis (1). In response to endothelium damage, platelets rapidly bind to the extracellular matrix, leading to platelet activation, clot formation, and the prevention of excessive bleeding. Platelet activation and clot formation lead to rapid energy production and utilization, as demonstrated by increased glucose uptake (2), glucose oxidation (3), glycogen utilization (4), lactic acid production (5), and mitochondrial respiration (6). This dramatic association between glucose metabolism and platelet activation is not well understood and raises questions integral to platelet function.

Is the relationship between glucose metabolism and platelet function correlative or causative? In vivo and in vitro hyperglycemic conditions lead to increased platelet glucose metabolism (7), activation and thrombosis (8). In vitro studies of platelets using inhibitors of glucose uptake and metabolism have demonstrated that glucose metabolism is essential for platelet activation (9, 10). This may be of particular importance because patients with T1DM and T2DM display increased thrombosis (11). Diabetes-mediated increased thrombosis has been attributed to multiple components of the thrombosis activation cascade, including increased platelet activation, vasculature dysfunction, activation of the complement system (12), and systemic inflammation (13). However, to date, no studies have evaluated the contribution of altered in vivo glucose metabolism on platelet function.

Platelet anatomy

Platelets are produced in megakaryocytes, which, in adults, are primarily located in the bone marrow. Although platelets lack nuclei, megakaryocytes do not, imparting platelets with large stores of RNA that can be translated while platelets are circulating or activated (14). Platelets also contain organelles, including mitochondria, rough endoplasmic reticulum, dense tubular system (smooth endoplasmic reticulum), and the open canicular system (which are invaginations in the platelet membrane) that aids in increasing membrane surface area (Figure 1.1) (15). In addition, platelets contain three different storage vesicles: α-granules (~40-60/platelet), δ-granules (~3-8/platelet), and lysosomes (15, 16), each of which contains diverse signaling molecules.

α-Granules are the most abundant granules in platelets, containing up to 300 signaling proteins such as PF4, RANTES, VEGF, PDGF, TGF, and many others (17). Upon activation, α-granules fuse with the plasma membrane and release their cargo (Figure 1.1). α-Granule proteins have been shown to play significant roles in multiple disease states, including malaria (18), angiogenesis (19, 20) , cancer (21, 22), and wound healing (23). In addition to cargo release, α-granule fusion with the plasma membrane leads to translocation of α-granule membrane proteins to the plasma membrane. The α-granule membrane proteins P-selectin and GLUT3 have both been shown to translocate to the plasma membrane following degranulation. This is important because P-selectin is a cell adhesion molecule that can bind to and recruit leukocytes to the sites of inflammation (24), as well as activate immune cells (25). α-Granule membrane proteins and cargo play diverse roles in thrombosis, angiogenesis, immune function, as well as other physiological functions (19).

δ-Granules contain small signaling molecules like ADP, ATP, NADH, polyphosphates, serotonin, and others (16). Following δ-granule release, ADP, ATP, and serotonin release can function in an autocrine or paracrine manner, amplifying platelet activation. In addition, release of these contents may facilitate activation of immune or endothelial cells, resulting in

inflammation or thrombosis.

Lysosomes are scarce in platelets, and releases of lysosome proteins are thought to contribute to clot remodeling. Interestingly, a recent study of a platelet-specific knockout of PIKfyve, an essential lysosome protein, resulted in decreased thrombosis, increased macrophage infiltration into multiple tissues, and death (26), therefore, the significance of platelet lysosomes is not completely understood and will require additional studies.

Platelet activation and signal transduction

Platelet activation is characterized by increased calcium flux, activation of the GPIIbIIIa protein complex, cytoskeleton rearrangement, and release of granules (Figure 1.2) (27), all of which lead to clot formation. Platelet activation can be facilitated through inside-out or outside-in mediated signaling. Outside-in signaling occurs when inactivated GPIIbIIIa protein complex binds to immobilized fibrinogen, resulting in a conformation change and activation of the GPIIbIIIa complex (28). Once GPIIbIIIa enters an activated conformation, the intracellular domain signals through SFKs, PI3K, Rho/Rac, and/or alternative pathways, eventually leading to platelet activation and spreading (29). Outside-in signaling not only allows for platelet accumulation at exposed fibrinogen, but also facilitates spreading and release granule content, which recruits additional platelets to the injury site and aids clot formation.

Inside-out signaling is characterized by agonist-induced receptor activation followed by signal transduction and platelet activation (30). Recent studies utilizing in vivo microscopy of clot formation have indicated that specific receptor-mediated activation plays a role in zonal partitioning of the clot. For instance, at the "core" of a clot, thrombin-mediated platelet activation is more important than in the "outer region," where ADP plays a more significant role (31). Although the exact consequence of the zonal distribution of platelet activation is not yet completely understood, these findings demonstrate that alternative platelet signaling pathways facilitate specific consequences in vivo.

Inside-out mediated platelet activation can be facilitated through multiple GPCRs (Figure 1.2). As its name implies, the thromboxane receptor activates in response to thromboxane A_2. This pathway is particularly important for amplifying platelet activation. Thromboxane A_2 is produced by activated platelets through COX-1 mediated metabolism of AA (32). Once synthesized, thromboxane A_2 is released and can activate the thromboxane receptor on platelets or several other cell types.

Platelets express the GPCRs PAR1, PAR3, and PAR4 in a species-dependent manner. Studies in humans have demonstrated an ethnicity-dependent expression, where the predominant receptor in white humans is PAR1, whereas blacks display increased signaling through the PAR4 (33). Importantly, murine platelets do not express PAR1, but facilitate the majority of PAR-mediated signaling through PAR4 (34). PAR class receptors are activated by proteolytic cleavage of peptides in their extracellular domain by thrombin, followed by the subsequent binding of the cleaved peptides to the cleaved receptor (34). This thrombin-sensitive receptor allows activation of the coagulation cascade, resulting in platelet activation and clot formation.

Platelets also contain the purinergic receptors $P2Y_1$, $P2Y_{12}$, and $P2X_1$ (35), which are GPCRs, and activation in response to ADP and ATP. Extracellular ADP and ATP are not typically found in the circulation, although release of δ-granules by platelets leads to high extracellular concentrations of ADP and ATP (36). These molecules are then able to act in an autocrine or paracrine fashion, leading to platelet aggregation and clot formation. This purinergic-mediated signal transduction amplifies platelet activation following stimulation (37).

Collagen can activate platelets through the tyrosine receptor kinase receptor GPVI. GPVI is a transmembrane glycoprotein containing an Ig domain similar to ITAM proteins, which associate with the FcRγ (38). GPVI binding of collagen results in receptor activation, cross talk with GPCR pathways, as well as SFK mediated signal transduction and platelet activation. Interestingly, platelet GPVI-mediated activation has been shown to facilitate unique responses in vascular permeability (39), rheumatoid arthritis (40), and response to inflammation (39).

Platelets express a variety of other receptors that contribute to platelet activation; however, their function will not be addressed in this dissertation.

Platelets as immune modulators

Classical studies of platelets have focused on their ability to activate in response to vascular damage and form a clot to prevent excessive bleeding. However, recent studies have implicated platelets as playing a major role in immune modulation. Studies of mice with platelet depletion following antiplatelet sera or anti-GPIbα antibodies demonstrated that platelets play an essential role in models of sepsis (41), malaria (18), transfusion-related acute lung injury (42), ischemia reperfusion-mediated stroke (43), KBXN-mediated rheumatoid arthritis (40), and many other inflammatory diseases.

In sepsis, platelet counts are one of the most reliable predictors of patient survival, with a low platelet count correlating with a poor prognosis (44). This correlation has been attributed to increased disseminated intravascular coagulation, leading to organ failure and mortality; however, mice depleted of platelets have decreased survival in sepsis models (41). This prosurvival function of platelets in sepsis is possibly due to the COX-1 mediated metabolism of AA to the anti-inflammatory prostanoids prostaglandin E_2 that inhibits macrophage activation and decreases IL-6 and TNFα production (41).

Sepsis is frequently accompanied by alterations in metabolism, including hypo- or hyperglycemia. Furthermore, glucose flux through the pentose phosphate pathway in platelets is known to regulate AA metabolism through $NADP^+$-NADPH cycling; however, to date no studies have examined this relationship. The effect of sepsis on platelet glucose metabolism is completely unknown, even though mitochondrial-mediated oxidative phosphorylation is known to increase in sepsis (45), possibly due to increased glucose oxidation. Therefore, future studies to investigate the relationship of plasma glucose levels, glucose metabolism, PPP flux, and modulation of AA metabolism are required to better understanding this enigmatic disease.

Platelets are essential for rheumatoid arthritis disease progression (40). Synovial fluid from patients with RA has significant enrichment of IL-1α and IL-1β positive platelet-derived microparticles, which are not observed in osteoarthritis patients. In a KBXN model of RA, mice deplete of platelets fail to develop disease. This effect is not simply due to the decreased platelet activation, because mice lacking cyclooxygenase ($Ptgs1^{-/-}$), thromboxane receptor, GPIIbIIIa ($Itgb3^{-/-}$), GPIbα, and ADP-P2Y12 all develop RA. However, mice lacking GPVI signaling displayed almost complete cessation of disease progression. This is due to the unique ability of GPVI to utilize ITAM signaling and generate IL-1α and IL-1β positive microparticles, which exacerbate RA disease progression (40).

Platelets play a major role in stroke disease progression. Strokes are frequently caused by a blood clot in the cerebral vasculature leading to decreased blood flow and brain ischemia, which cause hemorrhage. Although stroke is a leading cause of death in the US, there is currently only one approved therapeutic for treatment of ischemic stroke patients: tissue plasminogen activator, a thrombolytic (46). This lack of therapeutic options underlies the importance of better understanding the pathogenesis of stroke disease progression. Rodents subjected to MCAO, a model of cerebral ischemia/reperfusion injury, display a significant reliance on platelet function, although platelets do not facilitate the initial ischemic injury. Mice with platelets lacking functional binding proteins GPIb, GPIIb, GPIIbIIa, GPVI, or P-selectin (43, 47) all display decreased infarct size following MCAO. Additionally, Nbeal-deficient mice, which lack functional α-granules, display decreased infarct size in an MCAO model (23). However, mice with 100% loss of GPIIbIIIa receptor binding (48) or with platelet counts lower than 2.5% of control (49) have increased bleeding and die, indicating that some platelet function is required for normal hemostasis in this model.

Underlying all of these diverse and complex platelet functions is platelet metabolism. All platelet functions require metabolic energy for signaling and functional consequences. Therefore, we intend to gain a better understanding of platelets, metabolic pathways in order to determine

how energy metabolism may regulate platelet function.

Glucose transporters in platelets

SLC2 family genes encode glucose transporter proteins 1-14. GLUTs are integral membrane proteins, which transport substrates through facilitated diffusion (except GLUT13 which is a H^+ uniporter) (50). All GLUT proteins are composed of ~500 amino acid residues which form 12 transmembrane domains (51). These transporters can be categorized into 3 classes based on sequence similarity. Class I (GLUT1-4, 14), class II (5, 7, 9, 11) and class III (6, 8, 10, 12, 13). Class I glucose transporters have been extensively characterized and shown to be the major contributors to physiological glucose transport in mammals (52). Class II & III transporters can facilitate glucose transport under controlled conditions, however under physiological conditions, these transporters may be more important for the transport of non-glucose hexoses, fructose, ascorbic acid, and other substrates (50).

Class 1 glucose transporters serve as the gatekeepers of glucose entry into the cell. These transporters are expressed in every cell of the body, and their function can be regulated by the primary glucose requirements in the cell or hormonal control. For instance in insulin-sensitive cells, the majority of GLUT4 is localized to intracellular vesicles, and upon stimulation with insulin these vesicles fuse with the plasma membrane and facilitate glucose uptake. This spatial regulation of GLUT4 is not thought to apply to other class I glucose transporters, except GLUT3 in platelets (discussed below). GLUT1 is almost ubiquitously expressed throughout the body and is localized on the plasma membrane where it can facilitate basal glucose uptake. GLUT2 is also expressed on the plasma membrane, and its primary importance appears to be transport of glucose out of the liver following gluconeogenesis. Although GLUT3 is expressed in platelets and leukocytes, the majority of studies have investigated GLUT3s functions in neurons. In the brain, glucose concentrations are generally lower than other vascularized tissue, therefore it is thought that neurons utilize GLUT3s low Km for glucose uptake. Class I glucose transporter facilitates

the majority of glucose transport into cells and displays diverse function across tissue types.

Platelets express GLUT1 and GLUT3. Transcript and protein analysis has excluded other class I glucose transporters, GLUT2 and GLUT4, in human and murine platelets. The physiological contribution of GLUT1 and GLUT3 to platelet function has only modestly been studied due to the lack of pharmacological and experimental techniques to delineate their functions.

The contribution of GLUT1 to platelet function is unknown. In addition to glucose transport, GLUT1 has the ability to transport mannose, galactose, glucosamine, and reduced ascorbate (53). GLUT1 mRNA is found in low abundance in both mouse and human platelets (54) (unpublished data); however, murine megakaryocytes (data unpublished) and immortalized human megakaryocyte cell lines MEG-01, DAMI, and CHRF display abundant quantities of GLUT1 mRNA (2). GLUT1 protein is also present in murine megakaryocytes, murine platelets (unpublished data) and human platelets (55), although with low expression. Currently, there is no published data indicating the localization of GLUT1 in platelets. Although the significance of large quantities of GLUT1 mRNA in megakaryocytes and low quantities expressed in platelets is unknown, this disparity may indicate that GLUT1 plays a significant role in megakaryocyte function.

The contribution of GLUT3 expression to platelet function is unknown. Under experimental conditions, GLUT3 displays a lower K_m for glucose transport than GLUT1. In addition to glucose, GLUT3 has also been shown to transport galactose, mannose, and xylose (56). GLUT3 mRNA is highly expressed in humans and murine platelets (57); of all mRNAs expressed in human platelets, GLUT3 mRNA is ranked in the top 94^{th} percentile (54). Mouse megakaryocytes (unpublished data) and immortalized human megakaryocyte cell lines MEG-01, DAMI, and CHRF all revel the presence of GLUT3 mRNA (2). Interestingly, in humans, black individuals have decreased GLUT3 mRNA expression compared to whites (54). GLUT3's physiological function is thought to be regulated by its subcellular localization (58). Immunogold

labeling of GLUT3 in resting platelets indicates that 85% of GLUT3 is expressed in α-granule membranes and roughly 15% is expressed in the plasma membrane. Upon stimulation with thrombin, platelets degranulate and the α-granule membranes fuse with the plasma membrane, leading to GLUT3 incorporation into the plasma membrane (58). In concert with GLUT3 translocation, there is an increase in 2-deoxy-D-glucose uptake and a 1.5 fold increase in glucose V_{max}, indicating an increased ability of platelets to take up glucose upon activation (58). The significance of degranulation-mediated platelet glucose uptake is completely unknown, with the exception of clot retraction, which has been shown to be inhibited in vitro when glucose is removed from the media (59).

Platelet glucose pathways

Once glucose enters the platelet it can be utilized for multiple functions. Platelets contain all of the enzymes required to metabolize glucose through glycolysis, the pentose phosphate pathway, and polyol pathway, with multiple implications for platelet function, which are outlined below (Figure 1.3).

Glycolysis

Under resting conditions, washed platelets utilize roughly 55% of glucose derived from glucose uptake for glycolysis to be excreted as lactic acid (60), whereas an additional ~16% undergoes glycolytic conversion to pyruvate and is metabolized in mitochondria by oxidative phosphorylation (3). Metabolism-derived cycling of ATP is highly dynamic in platelets, occurring at a rate similar to neutrophils and leukocytes, and is 150 times higher than erythrocytes (61-63). The exact reason why platelets maintain this rapid energy cycling is unknown. Unlike nucleated cells, once platelets are circulating, they facilitate only minimal protein translation (64); additionally, recent reports demonstrated that platelets contain active proteasomes, whose function is ATP-dependent and essential for platelet function (65). One proposed rationale for this rapid energy cycling is that basal energy maintenance is required for getting priming for rapid

activation (66) and to maintain SERCA- and PMCA-mediated calcium maintenance.

Platelets stimulated with thrombin increase glucose uptake nearly 2-fold, this is in concert with increased glycogen utilization and glucose metabolism. Additionally, in the presence of thrombin, lactic acid production nearly doubles (5) and glucose oxidation increases 2.25-fold (3). This rapid increase in glucose metabolism is thought to fuel the estimated 3-fold increase in ATP utilization which occurs within the first 60 seconds of activation (61, 63). Upon activation, platelets increase actin polymerization, leading to dramatic cytoskeletal rearrangement, proteins synthesis, granule release, and integrin complex activation, all of which require substantial amounts of energy.

In order to understand if activation-induced glucose metabolism is essential for platelet activation, in vitro studies have treated platelets with various concentrations of glucose media or 2-DOG, a glucose analog, that competitively inhibits glucose transporter mediated uptake of glucose into platelets, and accumulates in the cytoplasm in the phosphorylated form, ultimately leading to toxicity (9). Interestingly, platelets incubated in the absence of glucose or presence of 2-DOG have decreased clot retraction (59) and reduced aggregation by over 50% (66). However, platelets pretreated with 2-DOG and the mitochondrial respiration inhibitor antimycin A show complete inability to activate in response to thrombin (10) or facilitate clot retraction (59), but antimycin A alone has no effect on platelet aggregation. This phenomenon underlies the important ability of platelets to utilize different substrates and metabolic pathways to produce metabolic ATP. Because platelet activation leads to multiple alterations in platelet function, the contribution of ATP to regulate specific platelet responses were undertaken. To do this, platelets were pretreated with 2-DOG and antimycin A for increasing lengths of time, then treated with thrombin, while simultaneously monitoring the platelet AEC (AEC= ([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP])). The AEC level was used to gain an estimation of the platelet energy state. Based on these experiments it was estimated that the platelet energy requirements of activation responses could be ranked as follows: aggregation< dense granule and

alpha granule secretion< acid hydrolase secretion< phosphatidylinositol breakdown, phosphatidate formation, and arachidonate liberation (62). Although these studies were unable to account for sequential energy demands, the activation markers were treated independently of one another. These studies demonstrate that different platelet functions may be alternatively regulated by ATP demand.

Glycogen

Glycogen is composed of hundred to hundreds of thousands of glucose molecules linked together to form heterogeneous polymers (67). Glycogen maintenance is a dynamic process: glycogen synthase facilitates the polymerization of glucose to glycogen while glycogen phosphorylase converts glycogen to glucose-1-phosphate for subsequent metabolism (67). Platelets contain large quantities of glycogen, which is visible by transmission electron microscopy at a density similar to skeletal muscle (4). It is not clear whether platelet glycogen stores are generated de novo once in the circulation or if megakaryocytes package glycogen into platelets prior to release.

Megakaryocytes contain large concentrations of glycogen fluctuating throughout development, with the largest concentration of glycogen present just prior to pro-platelet formation (68, 69). Following platelet formation, megakaryocyte glycogen is absent (69). It is unclear whether megakaryocytes consume the glycogen stores for energy to generate platelets or if the glycogen is packaged into the nascent platelets. Additionally, incubations of washed platelets with C^{14}-glucose leads to a significant accumulation of C^{14}-glucose into glycogen stores (70). Incubations with C^{14}-citrate and C^{14}-pyruvate lead to the accumulation of C^{14}-glycogen, indicating the presence of glyconeogenesis, but the rate of accumulation was quite low (70). Although platelets have the ability to maintain their glycogen stores, the mechanism by which glycogen accumulated in platelets remains unknown.

Thrombin-mediated platelet activation leads to the utilization of glycogen stores (4),

which is facilitated through GSK and AKT signaling (27), leading to inhibition of glycogen synthase and decreased glycogen synthesis. Additionally, platelets express neuronal, muscle, and liver-type glycogen phosphorylase, which activates in response to decreased cellular ratios of ATP/ADP and results in glycogen breakdown (4). In the absence of glucose and in the presence of CN, a mitochondrial electron transport chain inhibitor, glycogen stores can largely maintain platelet ATP levels for up to 40 minutes (70). Thus activation-mediated glycogen utilization may play an important role in energy utilization for platelet activation.

Pentose phosphate pathway

Platelets are capable of metabolizing glucose through the PPP, which is directly linked to NADPH biosynthesis and GSH cycling (71). NADPH and GSH have been shown to be essential regulators of platelet function (72-75). Interestingly, incubations of platelets AA lead to a nearly 10-fold increase in PPP flux. This increase in PPP flux was independent of COX-1-mediated AA metabolism, but is facilitated through an NADPH-dependent step of the lipoxygenase metabolic pathway. Importantly, lipoxygenase-mediated metabolism of AA can lead to the formation of multiple HETEs and leukotrienes, facilitating important roles in platelet activation, immune response, and inflammation (76). Therefore modulation of PPP flux may be a direct link between glucose metabolism, ROS production, and platelet activation. Future studies investigating the role of PPP-mediated glucose metabolism and platelet function are needed to better understand this relationship.

Polyol pathway

Platelets can metabolize glucose to sorbitol and fructose through the polyol pathway (77). This process requires aldose reductase to convert glucose to sorbitol in an NADPH-dependent manner, at which point sorbitol can be metabolized to fructose through sorbitol dehydrogenase, which reduces NAD+ to NADH and finally fructokinase can phosphorylate fructose-yielding fructose-6-phosphate, which can then proceed through glycolysis (78). Under basal conditions,

the significance of this pathway is unknown; however, platelets incubated in high-glucose (25mM) media display increased aldose reductase activity (77) and, when treated with collagen, exhibit increased aggregation and P-selectin surface translocation, which is reversible in the presence of aldose reductase inhibitors (79). This is possibly due to increased unscavenged ROS due to increased GSH-GSSG cycling because of increased utilization of NADPH by aldose reductase. Although that link between aldose reductase and ROS remains to be proven, it is known that treating platelets with N-acetyl cysteine, a GSH precursor, leads to inhibition of platelet activation (74). Aldose reductase transgenic mice with T1DM display increase in vivo thrombosis and in vitro aggregation, which is associated with increased intracellular ROS (79), and was decreased in vitro by administration of aldose reductase inhibitors. Many questions still remain to understand whether aldose reductase activity is dysregulated in disease states, and whether glucose flux through this pathway can alter platelet function and NADPH levels.

Hexosamine biosynthetic pathway

Glucose flux through the hexosamine biosynthetic pathway can regulate posttranslational protein modification by O-GlcNac in platelets. In vitro murine platelets treated with O-GlcNacylation inhibitors displayed no alteration in aggregation or activation, suggesting that there might be no significant role of O-GlcNac in platelet activation (80). However, the in vivo contribution of O-GlcNac has not been evaluated, nor have the consequence of protein modifications in megakaryocytes and the effect of this modification on long-term function.

Platelet dysfunction in diabetes

It is well established that patients with T1DM and T2DM have increased thrombosis, microvascular, and macrovascular disease (12, 81). Patients with T1DM (82) and T2DM (83) display increased urinary secretion of 11-dehydro-thromboxane B_2, an in vivo marker of thromboxane A_2 production, which is a maker of platelet activation. Also, whole blood isolated from patients with T1DM contain increased platelet-monocyte aggregates, used as a marker of

increased in vivo activation and degranulation (84). In vivo patients with T2DM subjected to acute hyperglycemia using a hyperglycemic clamp display decreased bleeding time, increased sheer stress-induced platelet activation, as well as an increased 11-dehydro-thromboxane B_2 urinary secretion (85). Ex vivo platelets isolated from diabetic subjects demonstrate increased "stickiness" when incubated in a glass bulb (86), increased binding to fibrinogen (87), increased sCD40L release (84, 88), increased aggregation in response to ADP (87) and collagen (79), and, in mice, increased GPIIbIIIa activation in response to PAR4 peptide (89). These ex vivo studies demonstrate an inherent change in platelet physiology in diabetes.

To date only one study has investigated the effect of diabetes on glucose metabolism, and this was conducted in rats with T1DM. Interestingly, these rats had increased ex vivo platelet aggregation and accumulation of the glycolysis intermediates: glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-bisphosphate, 3-phosphoglycerate, 2-phophoglycerate, phosphoenolpyruvate, and pyruvate (7), indicating glucose metabolism is altered in the diabetic platelet. Because platelet activation is so tightly coupled to ATP energy homeostasis, alterations in glycolysis may lead to more excitable platelets, and future studies are required to investigate this relationship.

Summary and research objectives

In summary, platelets facilitate complex physiological functions, including regulation of thrombosis, hemostasis, vascular permeability, and immune response. In vitro studies of platelets using metabolic inhibitors have indicated that platelet activation and clot retraction are dependent on energy metabolism. Additionally, increased glucose flux through the PPP, polyol pathway, and glycolysis have been correlated with altered platelet function in disease. Therefore, in order to better understand the contribution of glucose metabolism to platelet function we generated mice with platelet-specific knockouts of GLUT1, GLUT3, or both. Utilization of these models will allow us to determine the contribution of glucose metabolism to platelet function in vivo, and

test the hypothesis that glucose metabolism is essential for platelet function.

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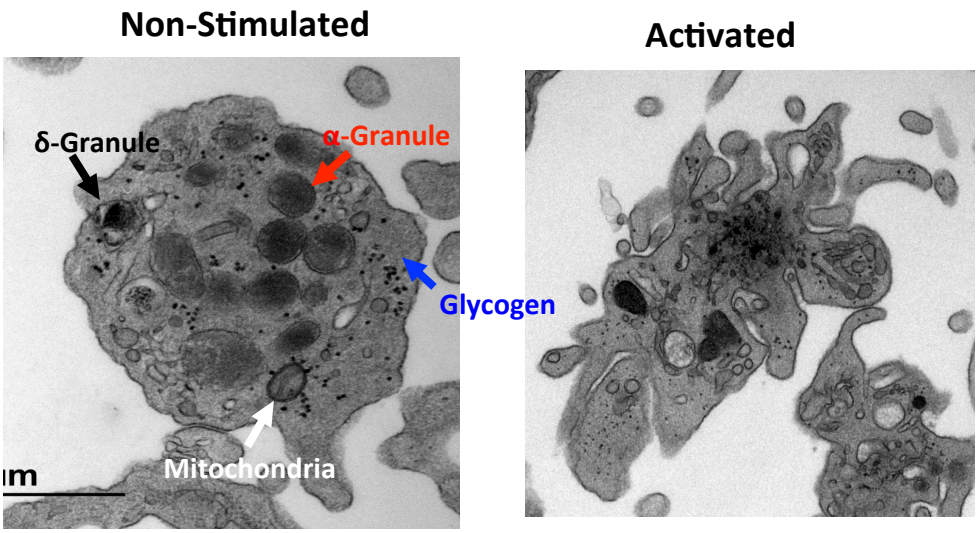

Figure 1.1 Murine platelet ultrastructure. Transmission electron micrographs of murine platelets under non-stimulated or PAR4 stimulated conditions. Major organelles are highlighted.

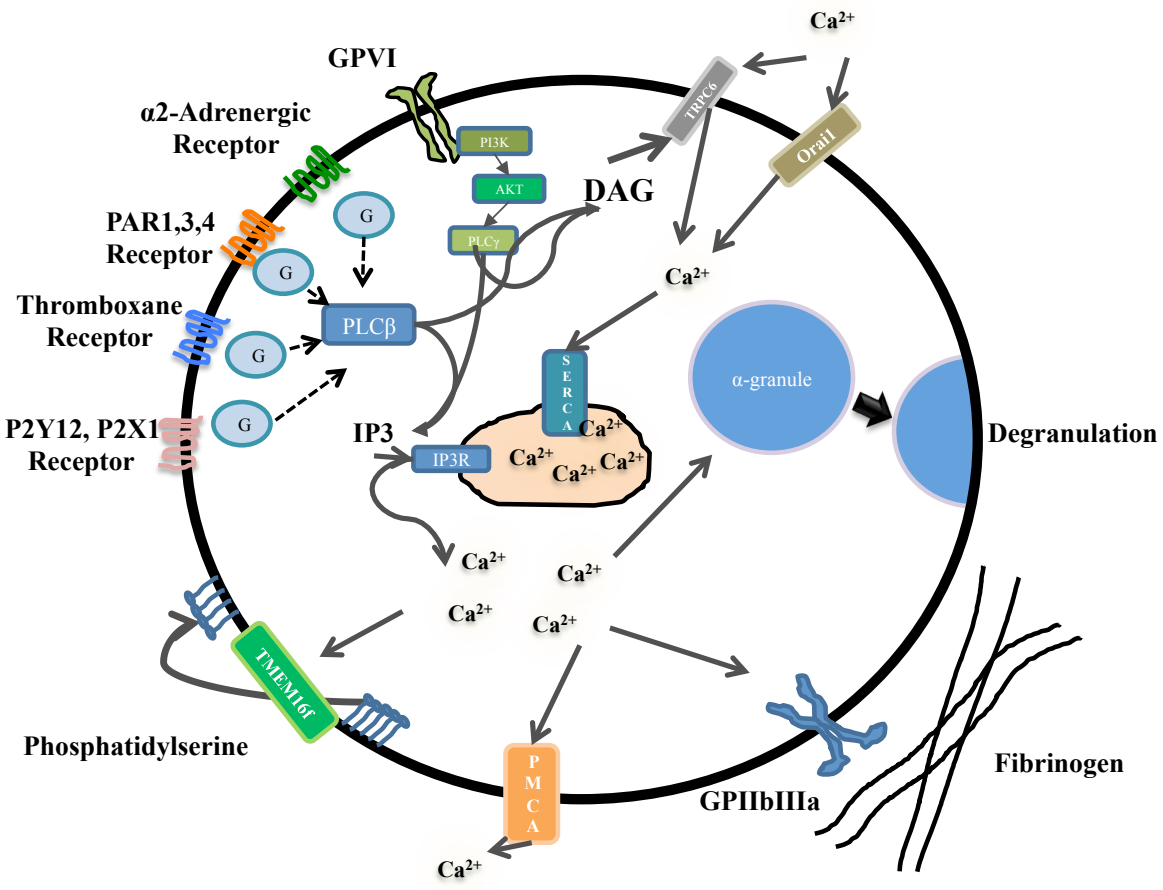

Figure 1.2 Platelet activation and calcium signaling. Agonist-mediated activation of receptors leads to PLC generation of IP3 and DAG. IP3 can then activate IP3 receptors which release stored calcium. In addition DAG can activate TRPC6 channels and facilitate calcium import from the extracellular environment. Increased cytoplasmic calcium flux then leads to the activation of the GPIIbIIIa complex, degranulation, and phosphatidylserine exposure to the outer leaflet of the plasma membrane.

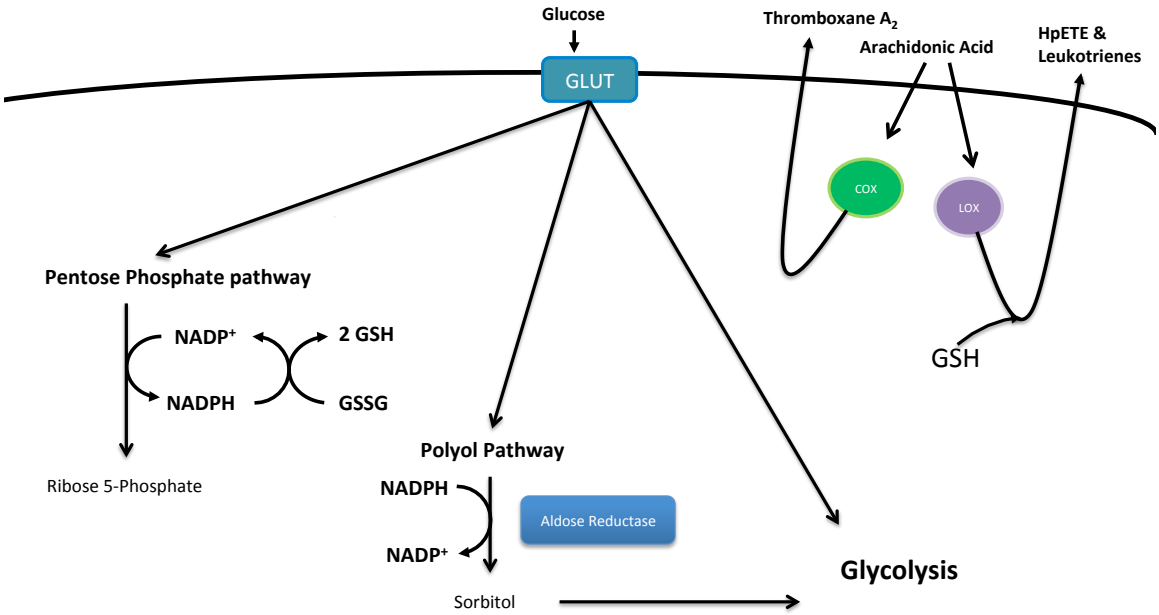

Figure 1.3 Cellular glucose metabolism. Glucose flux through the pentose phosphate pathway can generate NADPH and GSH, while glucose flux through the polyol pathway can utilize NADPH. Additionally Lipoxygenase (LOX) mediated metabolism of arachidonic acid can utilize GSH for the formation of HpETEs and leukotrienes.

CHAPTER 2

GLUCOSE TRANSPORTER 3 IS REQUIRED FOR INTRAGRANULAR GLYCOLYSIS AND PLATELET ACTIVATION

Abstract

Upon activation, platelets increase glucose uptake, glucose oxidation, lactic acid production, and consume stored glycogen. This correlation between glucose metabolism and platelet function is not well understood and even less is known about the role of glucose metabolism on platelet function in vivo. For glucose to enter a cell it must be transported through glucose transporters. Here we evaluate the contribution of glucose transporter 3 (GLUT3) to platelet function, using a platelet-specific knockout of GLUT3. In platelets, GLUT3 is localized primarily on α-granule membranes. Following activation, platelets degranulate and GLUT3 translocates to the plasma membrane, which is responsible for activation-mediated increased glucose uptake. Additionally, under basal conditions, GLUT3 facilitates glucose uptake into α-granules to be utilized for glycolysis, which aids in platelet degranulation. In vitro loss of GLUT3 leads to decreased platelet activation, degranulation, and spreading. In vivo, GLUT3 deletion leads to increased survival in a collagen/epinephrine model of pulmonary embolism and in a K/BxN model of autoimmune inflammatory disease, GLUT3 knockout (GLUT3-KO) mice display decreased disease progression. Together, these data indicate that GLUT3-mediated glucose utilization is integral for platelet function.

Introduction

Platelets are small anucleate cells generated by megakaryocytes that circulate in the blood with the primary function of hemostasis. Stimulation of platelets leads to activation, adherence to matrices, aggregation, and secretion of humoral mediators (1). Although once thought to be simple cellular fragments, platelets are now known to contain large amounts of RNA, synthesize proteins, and facilitate signaling in multiple disease states independent of thrombosis (2). These elaborate functions require energy (3). Platelet activation leads to increased glucose uptake, lactic acid production, and utilization of the large stores of glycogen (4, 5). Additionally, platelet activation is estimated to increase rates of ATP consumption by ~ 2-fold (4, 6).

Platelets express glucose transporter 1 (GLUT1) and the more abundant glucose transporter 3 (GLUT3) (7), which mediate glucose entry into the platelet via facilitative diffusion. Additionally, RNASeq analysis as well as proteomic analysis did not reveal GLUT2 or GLUT4 transcripts or proteins (8-11). Interestingly, roughly 85% of GLUT3 protein is localized to α-granule membranes, and the remaining 15% is found in the plasma membrane (12). Platelet degranulation leads to the fusion of α-granule membranes with the plasma membrane, which results in an increase in transporter available on the plasma membrane. This has been suggested to be the cause of increased glucose uptake following activation. The precise mechanism for why metabolism increases so dramatically following stimulation is unknown. It is plausible that post-activation glucose uptake may play a role in platelet activation. However, platelet degranulation in conjunction with GLUT3 translocation to the plasma membrane may continue to provide glucose for other purposes, such as clot retraction after the activation-mediated energy requirements are completed. Therefore, it is imperative to better understand the consequence of activation-mediated glucose metabolism on platelet function.

Currently the only functional studies of postactivation glucose metabolism revealed that in vitro, glucose is required in the media for clot retraction (13) and inhibiting platelet mitochondrial respiration and glycolysis blocks platelet aggregation (14). Even less is known

about the consequence of metabolism on platelet function in vivo, except for observations showing systemic administration of glucose leads to increased platelet activation (15, 16). However, it is unclear whether these effects are directly attributed to platelet metabolism, or could be the result of systemic responses.

To understand the contribution of glucose metabolism to platelet function we generated a platelet-specific knockout of glucose transporter 3 (GLUT3). This model allowed us to determine the exact contribution of glucose metabolism to platelet function in vivo, as well as the consequence of postactivation glucose uptake.

Results

Deletion of GLUT3 reduces glucose metabolism in platelets

To evaluate the contribution of glucose metabolism to platelet function we generated mice with platelet-specific knockout of GLUT3. Platelet-specific knockout of GLUT3 was verified via western blot analysis of lysates from GLUT3-KO platelets (Figure 2.1A). In addition, no compensatory increase in GLUT1 protein was observed. Basal total cellular glucose uptake was reduced by 23% in GLUT3-KO platelets compared to littermate controls (Figure 2.1B). Similar to human platelets (17, 18), thrombin elicited an increase in glucose uptake in control platelets, but this effect was abolished in GLUT3-KO platelets (Figure 2.1B). These data are consistent with the proposed model that translocation of GLUT3 to the plasma membrane facilitates postactivation glucose uptake (12).

Although glucose uptake was decreased in GLUT3-KO, both basal and thrombin-mediated glycolysis rates were unchanged (Figure 2.1C). However, following administration of mitochondrial inhibitors, GLUT3-KO platelets failed increase glycolysis to the same extent as observed in control platelets. These data suggest that unstimulated GLUT3-KO platelets under physiological conditions may sustain glycolysis via GLUT3-independent mechanisms such as increased glycogenolysis.

Metabolomics profiling of freshly isolated, quiescent platelets revealed that the pentose phosphate pathway (PPP) and glycolysis were modestly impacted by deletion of GLUT3. However, polyol pathway intermediates fructose (57.9±5.5 AUC vs. 28.0±5.8 AUC p=0.005) and sorbitol (31.9±8.3 AUC vs. 8.2±1 AUC p=0.03) were significantly reduced (Figure 2.1D). Importantly, glycogen content was decreased ~3-fold in GLUT3-KO platelets (Figure 2.1D).

Glucose metabolism in GLUT3-KO megakaryocytes was unchanged

We then investigated if metabolic alterations in platelets arise from dysfunctional megakaryocyte metabolism. Megakaryocytes derived from GLUT3-KO mouse bone marrow demonstrated a reduction in GLUT3 protein content (Figure 2.2A). This was associated with no changes in basal glycolysis, and unlike GLUT3-KO platelets GLUT3-KO megakaryocytes displayed no impairment in glycolysis following administration of mitochondrial inhibitors (Figure 2.2B). In contrast to platelets, GLUT3-KO megakaryocytes displayed no alteration in glycogen content (Figure 2.2C). Together these data suggest that the metabolic alterations in platelets do not arise from dysfunctional glucose metabolism in megakaryocytes.

Glycogen content and platelet activation

It is unclear whether glycogen utilization is required for platelet activation, and because freshly isolated GLUT3-KO platelets displayed decreased glycogen content, we evaluated the contribution of glycogen to platelet function. Administration of the glycogen phosphorylase inhibitor CP-316819 led to a dose-dependent decrease in wild-type platelet activation (Figure 2.8). Control platelets incubated in the presence of CP-316819 demonstrated decreased CD62p surface translocation, whereas the pre-existing defect in GLUT3-KO platelets was not significantly further inhibited (Figure 2.3A). We next evaluated the contribution of glycogen to platelet activation by loading or depleting glycogen stores respectively, following incubation in the presence or absence of glucose for 2 hours (Figure 2.3B). These glycogen-depleted and glycogen-rich platelets were then monitored for their ability to degranulate, as marked by CD62p

geo. MFI. Glycogen content did not influence baseline activation. Following stimulation with thrombin or PAR4 peptide all platelets demonstrated increased CD62p surface translocation (Figure 2.3C). Par4- and thrombin-stimulated GLUT3-KO platelets displayed decreased degranulation compared to controls under all metabolic conditions, despite equivalent degrees of glycogen loading or depletion relative to control platelets (Figure 2.3C). Depletion of glycogen in control platelets decreased thrombin-mediated degranulation with or without co-incubation with glucose at the time of stimulation (Figure 2.3C), suggesting that glycogen is required for platelet degranulation independent of extracellular glucose. Although a similar trend was observed, no significant additional defect in platelet function was observed between glycogen-rich and glycogen-depleted GLUT3-KO platelets when stimulated in the presence of extracellular glucose. However, GLUT3-KO platelets depleted of glycogen, when stimulated in the absence of extracellular glucose, exhibited decreased degranulation compared to glycogen-rich GLUT3-KO platelets stimulated in the absence of extracellular glucose (Figure 2.3C). These data suggest that GLUT3-KO platelets are more reliant on extracellular glucose in addition to existing glycogen stores. Thus glycogen metabolism may contribute to physiological platelet function and GLUT3-KO platelets display an attenuated response to inhibition of glycogenolysis, because of reduced glycogen stores in circulating platelets. Taken together, platelet dysfunction in GLUT3-deficient platelets might not only reflect decreased glucose uptake upon activation and reduced glucose availability from glycogen, but additional defects resulting from long-term reduction in GLUT3.

GLUT3-KO platelet activation and α-granule release are reduced

To determine the impact of GLUT3 deletion on agonist-mediated platelet activation, flow cytometric analysis of diluted whole blood was performed and GPIIbIIIa activation (JonA binding) and α-granule release (CD62p surface binding) monitored. GLUT3-KO platelets did not display changes in baseline activation. However, following stimulation with high concentrations of Par4 peptide, the thromboxane A_2 analog U46619 and convulxin, GLUT3-KO platelets

exhibited reduced GPIIbIIIa activation (Figure 2.3D) and CD62p surface translocation (Figure 2.3E). To determine if these changes were accompanied by impaired α-granule cargo release, the releasate from PAR4-peptide-stimulated platelets were evaluated for the protein content of pro-angiogenic factors. The majority of the angiogenesis proteins assayed were reduced in GLUT3-KO releasates (Figure 2.4A), although a subset of proteins was unchanged. In addition, ELISA quantification of releasate from thrombin-stimulated GLUT3-KO platelets exhibited reduced levels of PF4 (Figure 2.4B), and this decrease was not attributable to decreased total Pf4 content (Figure 2.4B). Platelet ultrastructure was also evaluated. Under basal conditions, there were no differences in α-granule, δ-granule, or mitochondrial number in nonstimulated platelets relative to controls (Figure 2.4C-E). However, following stimulation with Par4 peptide, GLUT3-KO platelets displayed a significant accumulation of partially released α-granules (Figure 2.4C-E). Because of the inability of platelets to activate in response to agonists in solution, we next examined static adhesion. Platelets were incubated on fibrinogen or collagen and treated with or without thrombin. GLUT3-KO platelets exhibited significantly impaired platelet spreading conditions (Figure 2.4F and G). Thus GLUT3-KO platelets display a degranulation defect.

GLUT3 facilitates platelet α-granule glycolysis

To investigate why deletion of GLUT3 did not alter basal or thrombin-mediated glycolysis rates in platelets, although degranulation was impaired, we considered the possibly that GLUT3 may mediate glucose utilization in intracellular organelles such as α-granules. Because GLUT3 is localized primarily to α-granule membranes in quiescent platelets (12), we hypothesized that GLUT3-mediated glucose uptake into α-granules facilitates intragranular glycolysis, which generates energy for degranulation. To test this hypothesis, we employed saponin permeabilization to distinguish between cytoplasmic and intragranular glycolysis. Following treatment of platelets with 10ng/mL saponin, we detected release of 90% of the platelet LDH into the washed supernatant (Figure 2.5A), which is a marker of plasma membrane

permeabilization. However, at this concentration, α-granules remained intact as determined by the absence of release of soluble α-granule protein PF4 (Figure 2.5B). Permeabilized GLUT3-KO platelets, with cytosolic proteins washed away, and incubated with C^{13}-1,6-glucose, displayed a ~2.5-fold decrease in C^{13}-lactic acid production (Figure 2.5C), indicating that α-granule glycolysis is regulated in part by via GLUT3-mediated glucose uptake.

Platelet dysfunction in vivo is context-dependent in GLUT3-KO mice

GLUT3-KO mice demonstrated no changes in hemostasis in vivo, as determined by tail-bleeding time (Figure 2.6A). Additionally, no differences were observed in a 10% ferric chloride carotid artery thrombosis model (Figure 2.6B) or in a stasis model of deep vein thrombosis following inferior vena cava ligation (Figure 2.6C). Because GLUT3-KO mice appeared significantly desensitized to collagen-induced activation, mice were subjected to collagen/epinephrine-induced pulmonary embolism. In agreement with in vitro activation data, GLUT3-KO mice displayed a significant increase in survival (Figure 2.6D). Together these data indicate that GLUT3 modulates platelet function in vivo, but only under specific circumstances.

Platelets play an essential role in disease progression of rheumatoid arthritis in a collagen-dependent manner, through platelet-derived microparticles and release of IL-1 (19). Because we observed a decrease in platelet activation and platelet spreading on collagen, we subjected these mice to a K/BxN model of autoimmune inflammatory arthritis. Interestingly, on transfer of arthritogenic serum, GLUT3-KO mice displayed a modest but significant and durable reduction in clinical index ($p<0.0001$) and ankle thickness ($p=0.0003$) (Figure 2.7A and 2.7B). Moreover, GLUT3-KO platelets displayed reduced IL-1α protein content (Figure 2.7C), without any change in *IL1A* transcript levels (Figure 2.7D), suggesting that the attenuated inflammatory response could be due in part to reduced IL-1α.

Discussion

These studies investigated the contribution of GLUT3-mediated metabolism to platelet function. Here we show that GLUT3 is responsible for ~20% of basal glucose uptake in quiescent platelets, and is largely responsible for the postactivation increase in glucose uptake (5, 12). RNASeq analysis of human and murine platelets as well as megakaryocytes indicate GLUT1 and GLUT3 are the only glucose transporters present in platelets (8-10). Therefore, our findings suggest that GLUT1 might be the major mediator of basal glucose uptake. Although basal glucose uptake was reduced in the GLUT3-KO platelets, no changes in basal glycolysis rates were observed. This might indicate an increase in glycogen consumption, a decrease in non-glycolysis-mediated metabolism, or a decrease in glucose oxidation into the mitochondria. Based on our findings, the most likely conclusion is that in the absence of GLUT3, glycolysis is maintained by increased glycogen utilization in conjunction with decreased polyol pathway metabolism. Indeed glycogen content was decreased 3-fold in the GLUT3-KO platelets and polyol pathway metabolites were significantly reduced. Additionally, mitochondrial respirations were unchanged under basal conditions in the GLUT3-KO platelets (Figure 2.9). Therefore, in the face of decreased glucose uptake, platelets may maintain glycolysis by increasing the utilization of stored glycogen and decreasing glucose flux through nonglycolytic pathways.

Upon activation, GLUT3 translocates from α-granule membranes to the plasma membrane (18), which is believed to mediate the increased glucose uptake. Our results definitively demonstrate that GLUT3 is required for increased glucose uptake. Because GLUT3 translocation occurs in concert with degranulation, postactivation glucose uptake might not be required for degranulation. However, intragranular glucose metabolism may regulate α-granule trafficking to the plasma membrane. Activation-mediated glycolysis was not impaired in GLUT3-KO platelets. We currently do not understand the significance of increased platelet glucose uptake following activation, but it is possible that postactivation glucose uptake may fuel additional platelet functions following activation. For example, in vitro studies indicate that in

the absence of glucose, clot retraction does not occur (13). The ability of GLUT3-KO platelets to undergo clot retraction was not assessed in vitro, however in an in vivo model of deep vein thrombosis, clot formation was unaffected. Although in vivo deep vein thrombosis and clot retraction are not the same, these data indicate that postactivation glucose uptake may not be essential for clot maintenance in vivo. Following activation, platelets produce microparticles, which play an essential role in rheumatoid arthritis disease progression as well other immune responses (20, 21). Microparticle formation is bioenergetically unfavorable. Following stimulation in vitro with potent agonists, mitochondria depolarize, and microparticles are generated hours later (22, 23). Thus glucose uptake and glycolysis might be the primary energy source that fuels microparticle generation and release. Although microparticle formation was not directly assessed in GLUT3-KO platelets, in a model of autoimmune inflammatory arthritis, which is dependent on microparticle formation, GLUT3-KO mice display decreased disease progression. Thus, although postactivation glucose uptake might not influence α-granule degranulation, it may mediate the release of other platelet-derived signaling mediators.

RNASeq analysis of murine megakaryocytes indicate the presence of both GLUT1 and GLUT3 transcripts (9), while significant quantities of GLUT3 are present in platelets, the GLUT1 transcript is expressed at very low levels (8, 10). These data may indicate that GLUT1 is present earlier in development and might be important for megakaryocyte glucose metabolism, whereas GLUT3 may be induced later. PF4 Cre deletes GLUT3 in the megakaryocyte. However in contrast to GLUT3-KO platelets, bone-marrow-derived megakaryocytes displayed no changes in basal or metabolic-stress-induced glycolysis or glycogen content. Thus GLUT3 might not be a major contributor to glucose metabolism in megakaryocytes. Therefore, the platelet dysfunction observed is likely to reflect the consequence of impaired GLUT3-mediated metabolism once platelets enter the circulation.

In vitro, GLUT3-KO platelets display significantly reduced degranulation. However, basal- and thrombin-stimulated glycolysis levels were unchanged. This led us to consider the

possibility that GLUT3 may play a role that is independent of cytoplasmic glucose uptake. Because subcellular localization of glycolysis can regulate cellular function (24), we considered the possibility that the enrichment of GLUT3 in α-granules might regulate intragranule glycolysis, which may be essential for generating the energy required for platelet degranulation. Proteomic analysis of α-granules, where GLUT3 was used as a specific marker of α-granules, as well as proteomic analysis of platelet releasates, indicates that all proteins required for glycolysis are present in α-granules (25-27). Saponin permeabilized platelets produced significant quantities of lactic acid, and deletion of GLUT3 reduced lactate efflux, confirming that GLUT3 facilitates glucose uptake into α-granules, where it can then be utilized for glycolysis. Although these permeabilization experiments did not differentiate α-granules from other intact intracellular organelles, GLUT3 has been described by multiple groups as specifically localized to α-granules (12, 26, 28).

We suspect that the significant reduction of α-granule release in the GLUT3-KO platelets is due to decreased intragranular glycolysis, which may locally generate ATP to provide the energy required for degranulation. ATP is an essential cofactor of degranulation. In permeabilized platelets devoid of cytosol, although degranulation can be initiated with the addition of calcium, ATP addition is an absolute requirement (29). An important ATP-requiring pathway involved in α-granule movement and degranulation is the ATPase N-ethylmaleimide-sensitive factor (NSF). In addition to NSF, multiple ATP-dependent proteins have been identified via α-granule proteomic surveys (26), which may require lumenal ATP for activation. Electron microscopic analysis of GLUT3-KO platelets stimulated with PAR4 demonstrates α-granules that are unable to release their cargo. We do not know if this is due to decreased plasma membrane fusion or altered α-granule trafficking. These observations support a model in which intragranular glycolysis would generate ATP in the α-granule lumen, making it readily available to proteins that may regulate α-granule trafficking and that also require lumenal ATP.

Glycogen is believed to play an essential role in platelet activation, given that platelet

activation occurs in concert with glycogen utilization (30). Surprisingly, very little is known about the relationship between glycogen content and platelet function. Studies of dense and light platelets in humans indicate denser platelets had more glycogen and were more responsive to agonists than the lighter platelets (31, 32). In support of these correlations, when we depleted platelets of glycogen there was a subtle decrease in the platelets' ability to activate relative to platelets containing normal glycogen content. Additionally, although glycogen stores were decreased ~3-fold in GLUT3-KO platelets, there was no reduction in circulating half-life, platelet number or mean platelet volume (data not shown), indicating glycogen content does not regulate platelet lifespan.

Deletion of GLUT3 was associated with a ~3-fold decrease in glycogen content. This could be a result of increased glycogen utilization or reduced glycogen synthesis in the α-granule. We are not aware of any reports that definitively demonstrate the presence of glycogen within α-granules. However, analysis of published isolated α-granules and platelet releasate proteomes indicate glycogen phosphorylase and glycolysis proteins are present in the α-granule (25-27). Additionally, radioisotope-labeling studies of glycogen dynamics in platelets have pointed to the possible presence of 2 glycogen pools (33). Thus, altered glycogen turnover (decreased synthesis or increased breakdown) might explain the fall in glycogen levels in the absence of GLUT3.

Deletion of GLUT3 dramatically reduced platelet function in vitro. GLUT3-KO platelets displayed reduced GPIIbIIIa activation and spreading on multiple matrixes. Although GLUT3-KO mice did not display abnormal bleeding, arterial thrombosis, or deep vein thrombosis, GLUT3-KO demonstrated significantly better outcomes in a pulmonary embolism model. It may be possible that the pulmonary embolism model provides a more sensitive index to monitor platelet function in vivo. However, it may also be possible that in vivo platelets may utilize alternative mitochondrial substrates to compensate for the decrease in α-granule energy production, many of which circulate in the plasma. Indeed, in the presence of glucose, glutamate, and pyruvate, GLUT3-KO platelets increased maximal mitochondrial respiration by 2.5 fold

(Figure 2.9). These data suggest that in vivo, platelets may dynamically regulate energy utilization, which may compensate for a deficit in α-granule glycolysis. The negligible impact of platelet GLUT3 deficiency on the regulation of thrombosis in vivo could have important therapeutic implications.

In a K/BxN model of autoimmune inflammatory arthritis, GLUT3 KO mice displayed a significant reduction in disease progression marked by clinical index. Platelets contribute to rheumatoid arthritis through the activation of the GPVI receptor, leading to IL-1α and IL-1β release along with microparticle production (21). Interestingly, KO platelets contained significantly decreased IL-1α protein content relative to controls. This decreases in IL-1α protein content was not accompanied by decreased *IL1A* mRNA. Unlike IL-1β, which is expressed upon activation (34), the mechanism of IL-1α synthesis in platelets is poorly understood (35). Our data demonstrated that megakaryocytes in culture maintain glucose metabolism, therefore it is highly suggestive that decreased basal glucose metabolism leads to post-transcriptional regulation of IL-1α in the circulating platelet. Although additional exploration of the mechanisms regulating IL-1α protein content are required, it is plausible that the reduction in IL-1α content may contribute to decreased arthritis disease progression. Because deletion of GLUT3 in platelets decreases inflammatory arthritis disease progression and increases survival following in vivo pulmonary embolism, GLUT3 could be considered a therapeutic target for conditions mediated via inflammatory mediators that are released from activated platelets.

Together these data indicate that GLUT3 may regulate platelet glycogen stores and represents an important mediator of α-granule glycolysis, which in turn may regulate α-granule secretion, platelet activation, and autoimmune inflammatory arthritis disease progression. These data underscore the significance of subcellular localization of metabolism and energy metabolism, which specifically regulates in vitro and in vivo platelet function.

Methods

Animals

Mice on a C57Bl/6 background were housed under standard conditions of temperature and lighting. Pf4 Cre transgenic mice were obtained from Jackson laboratories. GLUT3 floxed mice were obtained from the trans-NIH Knock-Out Mouse Project (KOMP) repository (www.komp.org), $Slc2a3^{tm1c(KOMP)Mbp}$. Floxed sites were inserted flanking Slc2a3 exon 7, and primers CCAACTTAAACACAATTGCCTGGTG and GGCTCACAATTACCCATAATGA were used for PCR identification of the GLUT3 floxed allele. GLUT3-KO mice were generated by crossing mice with homozygous GLUT3 floxed alleles to Pf4 Cre transgenic mice. Experiments were conducted on male mice between the ages of 8-14 weeks.

Platelet isolation

Whole blood was isolated from isoflurane-anesthetized mice through carotid artery cannulation into 1:20 acid-citrate-dextrose (ACD). Whole blood was then diluted with pipes saline glucose (PSG) and centrifuged at 120xg for 10 minutes. Platelet-rich plasma was then diluted with PSG with prostaglandin E_1 (PGE_1) (10nM) and centrifuged at 378xg x 10 minutes and centrifuged at 378xg x 10 minutes once more. When noted, platelets were incubated with Ter119 and CD45-labeled microbeads (Miltenyi Biotec, Auburn, CA) to deplete red blood cells and leukocytes. Following the isolation and centrifugation steps, platelets were allowed to recover for 30 minutes prior to experimental manipulation. Platelet counts were determined by Cellometer Auto M10 (Nexcelom Bioscience, Lawrence, MA).

Antibodies, arrays, and ELISAs

Antibodies for GLUT1 and GLUT3 immunoblots were obtained from EMD Millipore 07-1401 and AB1344 respectively. ELISAs for PF4 and IL-1α were provided by R and D Systems DY595 and DY400 respectively. Releasate from platelets treated with 150μM Par4 peptide was evaluated on an angiogenesis array obtained from R and D Systems (ARY015).

Glucose uptake

Washed platelets at a concentration of $2x10^8$/mL in DMEM with 1mM glucose were incubated with 10mM H^3-2-deoxy-D-glucose in the presence or absence of 1U/mL thrombin or 10μM Cytochalasin B. Samples were incubated for 10 minutes, washed three times in Stop Buffer (Ca^{2+} Free HBSS with 10μM Cytochalasin B) and solubilized in 1M NaOH. BCA protein analysis was conducted in one aliquot and the second subjected to scintillation counting.

Metabolite identification

Metabolomics analysis was conducted on liquid nitrogen flash-frozen platelets. Metabolites were isolated in 90% methanol solution, and analyzed as previously described (36). Glycogen was quantified in CD45 and Ter119 bead-depleted platelets using a Glycogen Assay Kit (Cayman chemical, Ann Arbor, MI).

Determination of platelet bioenergetics

Extracellular acidification rates and oxygen consumption of platelets were determined using the Seahorse XF24 analyzer (Seahorse Bioscience, North Billerica, MA) as previously described (37) following leukocyte and red blood cell depletion using Terr119 and CD45 micro beads. Platelets were seeded at a density of $1x10^8$ platelets/well in Seahorse XF24 Analyzer plates. Data were normalized to platelet counts.

Megakaryocyte cultures

Megakaryocyte cultures were conducted as previously described (9). Briefly, bone marrow from GLUT3-KO or control mice was flushed, and filtered through 70μM cell filters. Bone marrow was then cultured in DMEM with glucose, glutamate, and recombinant thrombopoietin (TPO) for 5 days. At day 5, megakaryocytes were enriched using a gravity BSA gradient of 1.5 and 3%. Megakaryocytes were then cultured overnight for experiments. Megakaryocyte culture purity was assessed by CD61 binding using flow cytometry.

Alpha granule glucose flux analysis

Washed platelets were suspended in buffer A (120mM sodium glutamate, 5mM potassium glutamate, 2.5mM EDTA, 2.5mM EGTA, 3.1mM $MgCl_2$, 1mM DTT, 20mM HEPES) and treated with saponin at a final concentration of 10ng/mL for 5 minutes. Permeabilized cells were then pelleted at 12,000xg for 2 minutes, supernatants were removed and platelets were resuspended in buffer A with 25mM C^{13}-1,6-glucose for 1 hour. Incubations were stopped by the addition of ice-cold methanol. Samples were then incubated at -20^{o}C for 1 hour and dried down under negative pressure and heat. Samples were then resuspended in pyridine spiked with the internal standard (5-fluoro-2-deoxyuracil) then derivatized with *N*-methyl-*N*-(tert-butyldimethylsilyl)-trifluoroacetamide + 1% tert-butyldimethychlorosilane and quantified using gas chromatography-mass spectroscopy.

Whole blood platelet activation

Fresh whole blood was diluted 1:10 in Ca^{2+}-free HT buffer. Diluted blood was then added to the specified agonist in the presence of JonA-PE, CD62p-FITC, and CD41-APC antibodies, at a final Ca^{2+} concentration of 5mM. Samples were then incubated at 37^{o} C for 10 minutes and fixed with 5 volumes FACs lysis buffer (Beckman Dickson, San Jose, CA). Samples were then analyzed using flow cytometry (LSR II, Beckman Dickson, San Jose, CA) gating for CD41 positive events.

Glycogen depletion

Washed platelets were incubated in DMEM in the presence or absence of 5mM glucose for 2 hour, at 37^{o}C with 5% CO_2. Platelets were then added to flow cytometry tubes with CD41-APC, CD62p-FITC and treated with the specified agonists for 10 minutes and the reaction stopped with FACs lysis buffer. The glycogen phosphorylase inhibitor CP-316819 suspended in DMSO was incubated with platelets for 30 minutes prior to treatment with thrombin and subsequent activation analysis.

Platelet releasate

Washed platelets were treated with 150μM Par4 peptide for 10 minutes at room temperature, spun down at 13,000xg for 2 minutes, and the supernatant was analyzed using an angiogenesis protein array (R & D Systems, Minneapolis, MN). For PF4 release assays, platelets were stimulated with 1U/mL thrombin for 10 minutes at room temperature, spun down at 13,000xg for 2 minutes, and the supernatant was analyzed by ELISA (R & D Systems, Minneapolis, MN).

Transmission electron microscopy

Washed platelets were incubated in DMEM in the presence or absence of 250μM Par4 peptide for 10 minutes at room temperature. Platelet stimulation was terminated by the addition of equal volumes of 4 % glutaraldehyde. Following 30-min glutaraldehyde incubation, platelets were gently centrifuged at 400xg for 10 minutes and resuspended in 4% glutaraldehyde. Samples were then processed as previously described for electron microscopy.

Platelet spreading

Washed platelets suspended in DMEM were incubated in fibrinogen or collagen IV coated chamber slides in the presence or absence of 0.5U/mL thrombin for 45 minutes under static conditions. Following incubations, platelets were fixed with 2% paraformaldehyde for 20 minutes at room temperature, washed, and stained with phalloidin-conjugated Alexa Fluor 488. Platelet spreading ratios were quantified as number of platelets bound divided by number of platelets spread.

RNASeq Analysis

CD45 and Terr119 bead-depleted platelets were submitted for RNASeq analysis as previously described (8). Data are represented as fragments per kilobase of exon per million fragments mapped (FPKM).

In vivo thrombosis

Collagen/epinephrine-induced pulmonary embolism: mice were injected i.v. with a mixture of 20μg/Kg epinephrine (Hospira Inc., Lake Forest, IL) and 430μg/Kg collagen (Chrono-Log, Columbia, MD) in PBS. Time of survival was determined by monitoring chest palpations, and time of death was determined as time when breathing ceased and did not return for 1 minute. Ferric-chloride-induced arterial thrombosis: mice were anesthetized with pentobarbital and mechanically ventilated. Exposed right carotid arteries were then treated with 1mm x 1mm whatman filter paper saturated with 10% ferric chloride for 3 minutes, rinsed with saline, and flow rate was monitored with a 0.5 PSB Doppler flow probe (Transonic Systems Inc., Ithaca, NY). Time to occlusion was monitored (38). Deep Vein Thrombosis was modeled using a stasis model of inferior vena cava ligation as previously described (39). Briefly, bowels were exteriorized by a midline laparotomy and the inferior vena cava was exposed by blunt dissection. A #6.0-silk suture was used to ligate the side branches and the inferior vena cava, inferior to the left renal branch. Animals were sutured close in a two layer fashion. 48 hours later, mice were euthanized and thrombus was harvested.

K/BxN-mediated autoimmune inflammatory arthritis

Mice were injected with K/BxN serum at day 0 and day 2. Ankle thickness and clinical index were monitored daily for 11 days as previously described (21). Data collection and analysis were conduced blinded to genotype.

Statistics

Data represented as mean ± standard error of the mean. Statistical analysis was conducted using GraphPad 6 and/or Microsoft office Excel 2011. t-test and ANOVA analysis were utilized when appropriate and following determination of normal distribution. Multiple comparisons were evaluated using a Tukey's analysis. Logrank (Mantel-Cox test) and Gehan-Breslow-Wilcoxon test were used for survival analyses. $P<0.05$ were considered significant.

Animal Studies

All animal studies were approved by the institutional animal care and use committee (IACUC) of the University of Iowa and the University of Utah.

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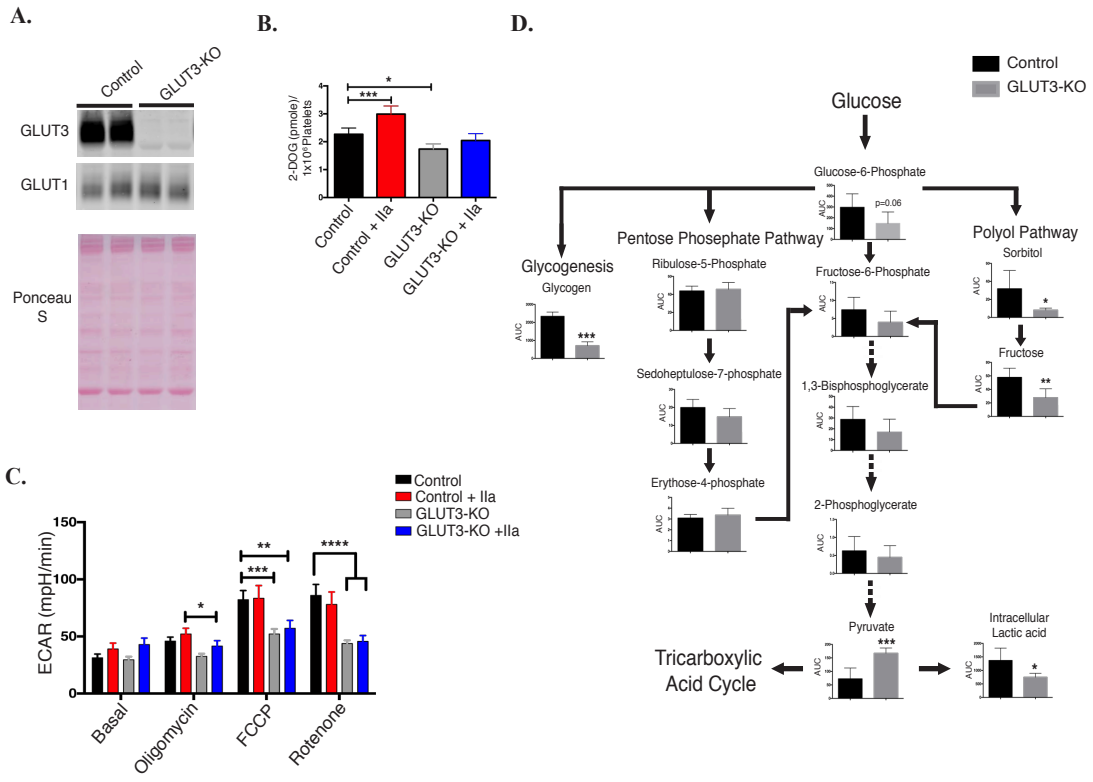

Figure 2.1. GLUT3 deletion reduces platelet glucose utilization. (A) Western blot analysis of GLUT3 and GLUT1 protein expression in platelet lysates from control and GLUT3 knockout platelets, n=6. (B) H3-2-deoxy-D-glucose (2-DOG) uptake in platelets was monitored in the presence or absence of 1U/mL thrombin (IIa), n=14. (C) Platelet glycolysis rate, as measured by the extracellular acidification rate (ECAR) was determined using a Seahorse XF24 analyzer. Platelet glycolysis rates with or without 1U/mL thrombin (IIa) were monitored under basal conditions and following treatment with the mitochondrial inhibitors: Oligomycin - ATP synthase inhibitor, carbonyl cyanide-ptifluoromethoxyphenylhydrazone (FCCP) - mitochondrial uncoupler, and rotenone, mitochondrial complex I inhibitor, n=5. (D) Metabolomics analysis of glycolytic intermediates in nonstimulated freshly isolated platelets were determined and normalized to platelet number, n=5. Glycogen analysis was determined fluorometrically by the enzymatic conversion of glycogen to glucose, n=6.*P<0.05, **P<0.01,***P<0.001; (2-way ANOVA followed by Tukey's multiple comparison post hoc test (B and C)); Student's t test (D).

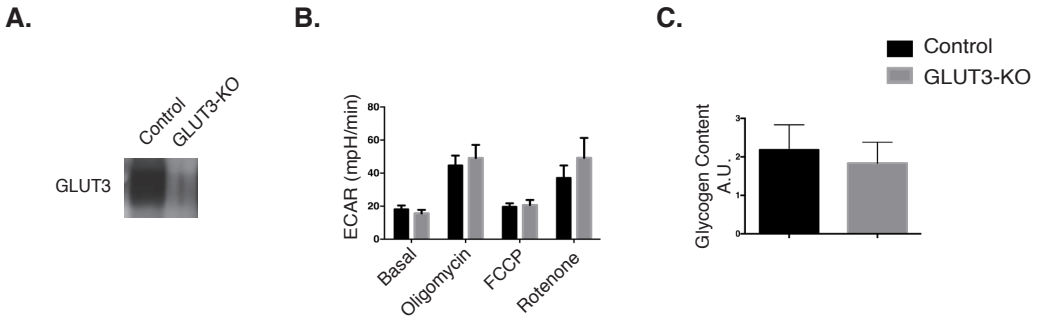

Figure 2.2. GLUT3 mediated metabolism in megakaryocytes. Bone-marrow-derived megakaryocytes were generated by culturing bone marrow for five days in thrombopoietin. A. Western blot analysis of megakaryocyte GLUT3 protein expression. B. Analysis of megakaryocyte glycolysis rate was measured as described in figure 1, n=6. C. Glycogen was evaluated fluorometrically by the enzymatic conversion of glycogen to glucose, n=6. (1-way ANOVA followed by Tukey's multiple comparison post hoc test (B)); Student's t test (C).

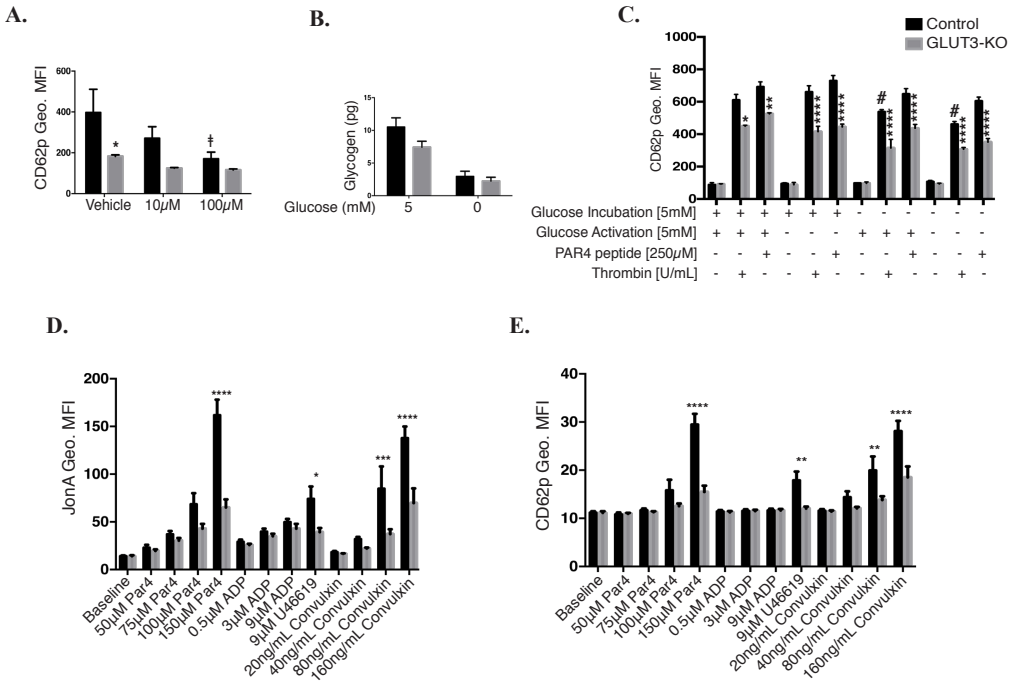

Figure 2.3. GLUT3 deletion leads to decreased platelet activation in vitro. A. Platelets preincubated with CP-316819 or vehicle at the designated concentrations were treated with 1U/mL Thrombin. B. Platelets incubated in DMEM in the presence of 5mM or 0mM glucose for 2 hours were assessed for glycogen content then subjected to activation studies (C). C. Platelets from panel B were incubated in the presence of vehicle, 250uM PAR4 peptide, or 1U/mL thrombin in the presence or absence of glucose and CD62p geometric mean fluorescence was monitored, n=4. D-E. Diluted whole-blood was treated with PAR4 peptide, ADP, U46619 and Convulxin at submaximal and maximal agonist concentrations. Platelets were monitored using flow cytometry gating for CD41 positive platelets with analysis of activated GPIIbIIIa as marked by (D), JONA geometric mean fluorescence (E), and CD62p geometric mean fluorescence, n=6. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control same treatment, #P<0.001 vs. Control + Glucose Incubation – Glucose Activation + Thrombin; (2-way ANOVA followed by Tukey's multiple comparison post hoc test (A-E)) ‡P<0.05 vs. vehicle same genotype (C).

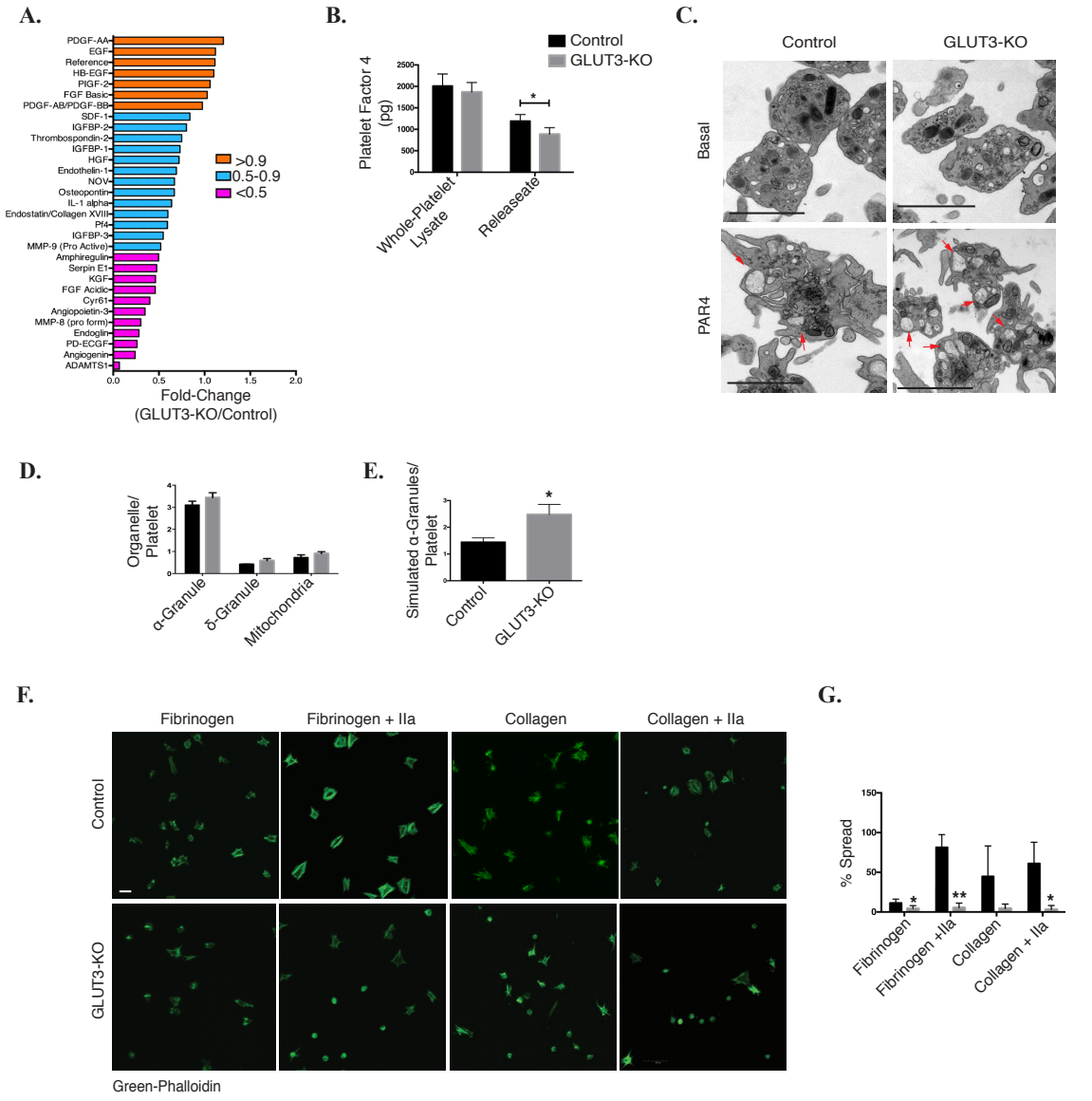

Figure 2.4. α-Granule cargo release and in vitro platelet spreading is reduced in GLUT3-KO platelets. A. Par4 peptide stimulated platelet releasate was monitored using a targeted angiogenesis protein array and expressed as % change relative to control, n=1. B. ELISA quantification of platelet factor 4 (PF4) in whole platelet lysates and releaseate from platelets stimulated with thrombin (1U/mL), n=9. C. Isolated platelets in suspension were fixed and analyzed using transmission electron microscopy. α-Granule, δ-Granule, and mitochondria were quantified and normalized per platelet. Platelets stimulated with 250uM PAR4 peptide in solution were also examined, with α-granules in the process of degranulation, depicted by red arrows; scale bar is 2μm. D, E. Numbers of structures per platelet were quantified, n=4. F. Washed platelets were incubated on collagen- or fibrinogen-coated chamber slides under static conditions in the presence or absence of 0.1U/mL thrombin (IIa), and stained with phalloidin (green) for cytoskeletal changes, scale 5μm, n=4. G. Spreading was quantified as summarized in the bar graphs. *P<0.05, **P<0.01; (2-way ANOVA followed by Tukey's multiple comparison post hoc test (D And G)). Student's t test (B And E).

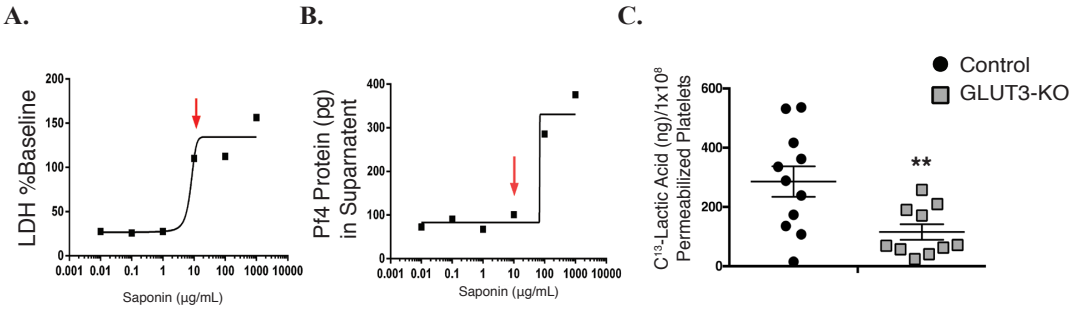

Figure 2.5. GLUT3 facilitates intragranular glycolysis. Saponin-permeabilized platelets were analyzed for A. LDH release and B. Pf4 Release. Platelets permeabilized with 10ng/mL saponin (red arrow), were incubated in the presence of C^{13}-glucose for 1 hour. C. Samples were then isolated in 90% methanol and C^{13}-lactic acid content determined by GC-MS, n=10. **P<0.01; Student's t test (C)

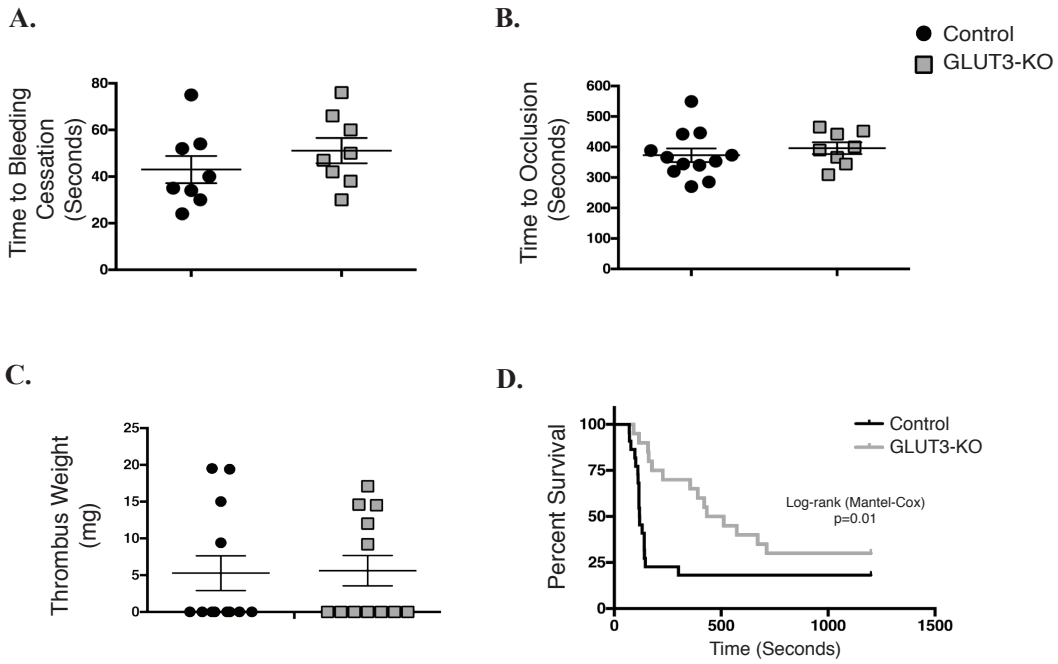

Figure 2.6. In vivo thrombosis. A. Tail bleeding was assessed by analysis of time to bleeding cessation following excision of 3mm tip of the murine tail, n=8. B. Mice were subjected to a 10% ferric-chloride-induced carotid arterial thrombosis, n=8. C. A stasis model of deep vein thrombosis was induced by inferior vena cava ligation and thrombus size determined. n=14. D. Survival curve following collagen/epinephrine induced pulmonary embolism, n=22. Student's t test (A-C); Log-rank (Mantel-Cox) test (D).

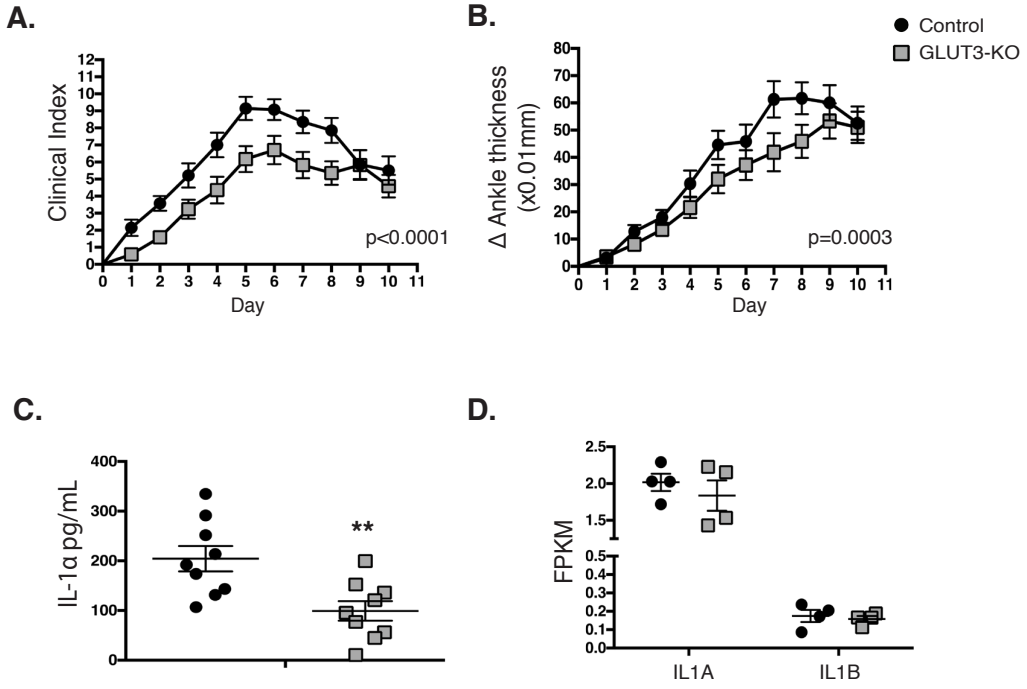

Figure 2.7. GLUT3-KO mice display decreased rheumatoid arthritis disease progression. Mice injected with K/BxN serum to precipitate rheumatoid arthritis were monitored for A. clinical progression and B. change in ankle thickness, n=14. C. Total IL-1α protein expression in platelets was assessed by ELISA, n=9. D. RNASeq transcript analysis of GLUT3-KO platelets, data represented as FPKM (fragments per kilobase of exon per million fragments mapped). *P<0.05, **P<0.01; (2-way ANOVA followed by Bonferroni correction (A and B); Student's t test (C-D).

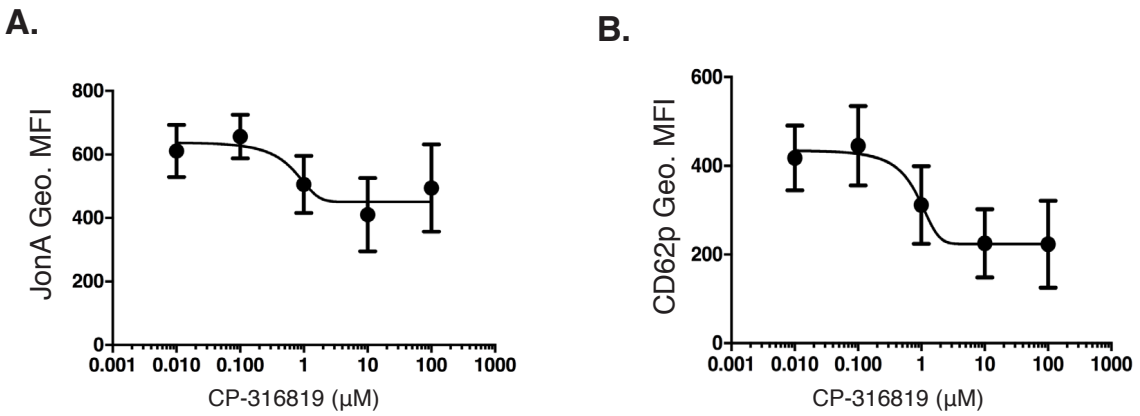

Figure 2.8. Dose response of CP-316819. Platelets from C57Bl6 wild-type mice were preincubated for 30 minutes with CP-316819 then stimulated with 1U/mL thrombin. A. JonA Geo. MFI and B. CD62p Surface translocation were monitored, n=3.

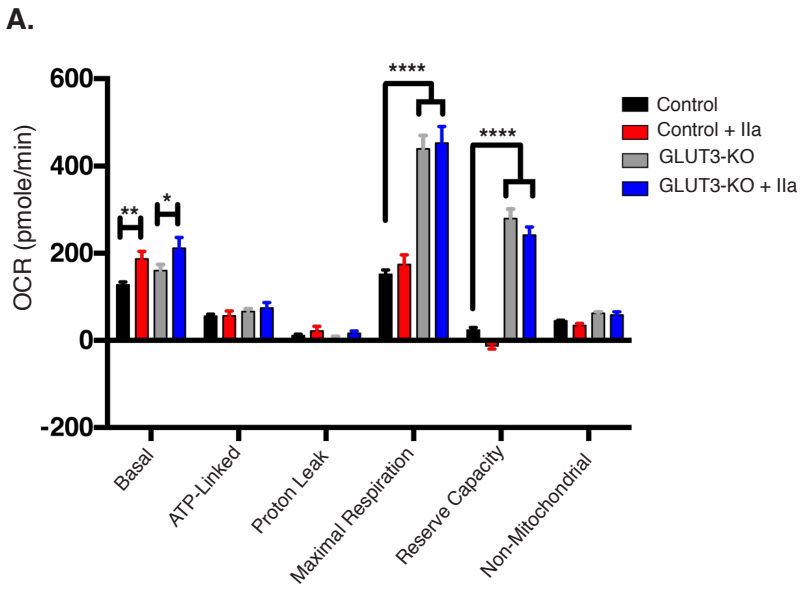

Figure 2.9. Mitochondiral respiration in platelets. A. Seahorse XF24 analysis of platelet mitochondrial respiration in the presence of 25mM glucose, plus 1mM glutamate, and 1mM sodium pyruvate in the presence or absence of thrombin 1U/mL (IIa), n=5.*P<0.05, **P<0.01,****P<0.0001; 2-way ANOVA followed by Tukey's multiple comparison post hoc test.

CHAPTER 3

GLUCOSE METABOLISM IS ESSENTIAL FOR PLATELET FUNCTION

Abstract

Platelet incubated under hyperglycemic conditions and platelets isolated from diabetic patients display increased activation. However, the contribution of glucose metabolism to platelet function is not well understood. Upon activation, platelets utilize stored glycogen and also increase glucose uptake, glucose oxidation, and lactic acid production. Cellular uptake of glucose is facilitated by SLC2A family glucose transporters, of which platelets express glucose transporter 1 (GLUT1) and glucose transporter 3 (GLUT3). To test the hypothesis that glucose metabolism is essential for platelet function in vitro and in vivo, we generated a platelet-specific knockout of both GLUT1 and GLUT3 by crossing mice expressing a Pf4-promoter-driven cre recombinase with mice harboring both GLUT1 and GLUT3 floxed alleles. GLUT1 and GLUT3 double knockout (DKO) platelets transport virtually no glucose, indicating GLUT1 and GLUT3 are exclusively responsible for platelet glucose uptake. Rates of glycolysis in DKO platelets were significantly reduced at baseline and failed to increase following treatment with the mitochondrial inhibitors oligomycin, FCCP, and rotenone. Interestingly, DKO mice display a significant reduction in platelet counts, circulating half-life, and decreased platelet regeneration following depletion with antiserum. Additionally, DKO platelets stimulated in vitro with PAR4 peptide, ADP, convulxin, and the thromboxane receptor agonist U46619 all display significantly reduced platelet activation determined by CD62p surface translocation, activation of GPIIbIIIa complex, and retention of α-granules. In vivo DKO mice subjected to tail bleeding or $FeCl_3$-induced

arterial thrombosis have significantly increased time to occlusion. Together these data indicate that glucose metabolism is essential for platelet production, maintenance, and function in vitro and in vivo.

Introduction

Patients with diabetes have increased thrombosis and platelet activation (1-3). In diabetic patients the contribution of endothelial dysfunction (1), systemic inflammation (4), and changes in the complement system have all been shown to contribute to increased in vivo thrombosis. In vitro studies have suggested a direct effect of glucose on platelet reactivity (5, 6). Studies of platelets from diabetic rats display increased glycolysis intermediates (7) as well as increased platelet activation. Platelets incubated under hyperglycemic conditions display increased activation in response ADP, arachidonic acid, thrombin, and collagen (6), indicating glucose can significantly influence platelet function. In vitro treatment of platelets with 2-deoxy-d-glucose (2-DOG), a glucose analog that is unable to be metabolized and competitively inhibits glucose uptake, impairs platelet activation (8). Additionally, upon activation platelets increase glucose uptake (9), glucose oxidation (10), lactic acid production (11), and utilize stored glycogen (12), suggesting an important role for glucose metabolism in platelet activation. Together, these in vitro studies reveal a direct relationship between glucose metabolism and platelet function.

Platelets express the facilitative glucose transporters GLUT1 (13) and GLUT3 (14). GLUT3 is primarily located in α-granule membranes and ~15% are localized in the plasma membrane. Upon degranulation, GLUT3 translocates to the plasma membrane and is believed to account for the increased glucose uptake following activation (14). To test the hypothesis that glucose metabolism is essential for in vitro and in vivo platelet function, we generated mice lacking GLUT1 and GLUT3 specifically in platelets.

Platelets play a major role in thrombosis and hemostasis. Therefore we investigated the ability of glucose-transporter-deficient platelets to function in bleeding and arterial thrombosis as

well as the contribution of glucose metabolism to platelet production and circulating half-life.

Results

Glucose metabolism is abolished in DKO platelets

To investigate the contribution of glucose metabolism to platelet function, both GLUT1 and GLUT3 were simultaneously deleted from platelets by crossing mice expressing a Pf4-promoter-driven cre recombinase to mice harboring homozygous floxed GLUT1 and GLUT3 alleles individually or in combination. Western blot analysis on protein lysates isolated from DKO and littermate control platelets (Figure 3.1A) demonstrated reduced GLUT1 and GLUT3 protein expression. Deletion of GLUT3 alone (GLUT3-KO) led to a ~22% decrease in basal glucose uptake and complete abolishment of thrombin-stimulated glucose uptake (Figure 3.1B), suggesting that GLUT3 is responsible for postactivation glucose uptake. Surprisingly, GLUT1 knockout (GLUT1-KO) platelets displayed no alteration in basal or thrombin-mediated glucose uptake. However, deletion of both GLUT1 and GLUT3 (DKO) led to a complete inhibition of glucose uptake into platelets, indicating that GLUT1 and GLUT3 are the predominant glucose transporters responsible for importing glucose into platelets and share overlapping functions.

Because glucose uptake was abolished in DKO platelets we hypothesized that decreased glucose uptake will result in decreased glycolysis and will result in a compensatory increase in mitochondrial respiration. To test this hypothesis we determined platelet extracellular acidification rate, a surrogate marker of lactic acid production, the terminal step of glycolysis. Under basal conditions DKO platelets had significantly reduced glycolysis rates compared to littermate controls (5.3±0.6 vs. 13.5±1.6) (Figure 3.1C). In human platelets thrombin-mediated activation results in increased lactic acid production (15), and, consistent with this, we observed that thrombin increased glycolysis by ~2-fold in control platelets. By contrast, DKO platelets were unable to increase glycolysis (Figure 3.1C). To explore potential adaptations to a glycolytic deficit that could sustain cellular bioenergetics, we assessed mitochondrial oxygen (O_2)

consumption in the presence of glucose, glutamate, and pyruvate. At baseline, O_2 consumption rates in DKO platelets were ~ 3-fold higher than control platelets (Figure 3.1D), suggesting increased utilization of glutamate or pyruvate by DKO cells. Of note, mitochondrial distribution in platelets remained unchanged (Figure 3.4C And D) in DKO platelets, suggesting a qualitative rather than a quantitative change in mitochondria from DKO platelets. Under basal conditions platelets obtain ATP via anaerobic and aerobic respiration (16). Thus DKO platelets appear to have become predominantly reliant on mitochondrial respiration for metabolic energy.

DKO mice are thrombocytopenic

DKO mice demonstrated reduced circulating platelet counts (1203±179 vs. 807±155k/dL, p=0.0009) (Figure 3.2A); however, platelet counts were unchanged in mice with GLUT1- or GLUT3-deficient platelets. To determine the cause of reduced platelet counts in DKO mice, we investigated platelet production. Administration of an anti-GPIbα antibody led to platelet depletion within 18 hours (Figure 3.2B). DKO mice required an additional 2 days for platelet recovery, suggesting that platelet biogenesis by DKO megakaryocytes might be impaired under conditions associated with increased platelet consumption. To further investigate whether DKO mice have dysfunctional megakaryocytes, cross-sectional analysis of femurs for megakaryocyte density was conducted. No significant alteration in megakaryocyte density or size was observed in DKO mice (Figure 3.2C And D). Moreover, the ability of bone-marrow-derived megakaryocytes to produce platelets in vitro was significantly reduced (Figure 3.2E And F). Thus glucose metabolism does not regulate late-stage megakaryocyte differentiation, although glucose metabolism does play a major role in platelet budding.

Phosphatidylserine exposure is increased in DKO platelets in response to metabolic stress

DKO platelets display decreased circulating half-life (Figure 3.3A). Because platelet clearance can be potentiated by increasing extracellular exposure of phosphatidylserine (PS) on

plasma membranes (17), we examined annexin v binding in DKO platelets. Control platelets incubated in DMEM with 5mM glucose had minimal annexin-v-positive platelets for up to 6 hours (Figure 3.3B). In contrast, greater than 25% of DKO platelets became annexin v positive (Figure 3.3B). Supplementation of the media with the mitochondrial substrates glutamate and pyruvate significantly reduced annexin v binding in DKO platelets (Figure 3.3B), suggesting that annexin v binding could be a marker of energetic stress.

Platelet annexin v binding has been shown to be facilitated by either mitochondrial depolarization leading to caspase 3/7 activation (18) or by activation of TMEM16F through increased cytoplasmic calcium concentrations ($[Ca^{2+}]_i$) (19). Following 6 hours incubation DKO platelets negative for annexin v demonstrated ~3-fold increase in mitochondrial potential, and annexin-v-positive platelets demonstrated an almost complete cessation of mitochondrial potential (Figure 3.3C). Surprisingly, following 1 hour of incubation, DKO platelets displayed significantly reduced caspase 3/7 activity relative to control, while at 6 hours this significant reduction was ablated (Figure 3.3D), indicating that relative to control, DKO platelets caspase 3/7 activity increased over the 6-hour incubation period. Following treatment with the BH3-mimetic ABT-737, DKO platelets did not demonstrate increased sensitivity marked by caspase 3/7 activity or PS exposure. Although 25% of DKO platelets were annexin v positive, ABT-737 induced annexin positivity and caspase 3/7 activation in 100% of platelets, with a dose response that was similar between DKO and WT platelets (Supplemental Figure 3.1A and B).

Platelets activation is decreased in DKO platelets

DKO platelets have a blunted ability to respond to agonists. In vitro analysis of diluted whole blood revealed that activation of DKO platelets, as determined by GPIIbIIIa activation (JonA geometric mean fluorescent intensity (geo. MFI)) (Figure 3.4A) and CD62p surface translocation (CD62p geo. MFI) (Figure 3.4B), was markedly decreased in response to PAR4 peptide, the thromboxane receptor agonist U46619, ADP, and convulxin. Because CD62p

translocation is a marker of α-granule degranulation, we further investigated α-granule release. Transmission electron microscopy (TEM) analysis of DKO platelets stimulated with PAR4 peptide in solution revealed retention of α-granules, consistent with decreased release (Figure 3.4C-E). In addition to these classical platelet markers, DKO platelets failed to expose PS on the plasma membrane following stimulation with thrombin or thrombin plus convulxin (Figure 3.5A) and displayed blunted GPIIbIIIa activation and CD62p surface translocation (Figure 3.5B And C). This activation-mediated PS exposure on the plasma membrane is mediated by calcium, the flux of which was also decreased following thrombin plus convulxin treatment (Figure 3.5D). Administration of the calcium ionophore A23187 increased annexin v binding to equivalent extents in DKO and control platelets (Figure 3.5D), indicating that mediates of PS exposure downstream of calcium, signaling are unaltered in DKO platelets. Platelet activation signal transduction converges on calcium flux that are reflected by the final markers of activation (20). Thus the lack of DKO platelets to increase calcium flux following activation through two distinct signaling pathways should blunt downstream processes. To evaluate if activation was impaired downstream of calcium signaling platelets were treated with a calcium ionophore ionomycin. Both activation of GPIIbIIIa and CD62p surface translocation were reduced in ionomycin treated DKO platelets (Figure 3.5E And F). Although the ability of DKO platelets to expose PS following ionophore treatment was unimpaired, activation was significantly blunted. Together these data suggest that glucose metabolism is essential for calcium signaling and facilitating platelet activation downstream of calcium flux.

Platelet glucose metabolism is essential for in vivo thrombosis

The contribution of platelet glucose metabolism to thrombosis and hemostasis in vivo was evaluated. In a tail-bleeding assay, DKO mice exhibited significantly longer time to bleeding cessation, with many mice failing to stop prior to assay completion (Figure 3.6A). Arterial thrombosis was also evaluated using 7.5% ferric chloride. DKO mice had significantly decreased

time to arterial occlusion relative to littermate controls, with many failing to occlude after 20 minutes of observation (Figure 3.6B). In a collage-epinephrine-induced pulmonary embolism model, DKO mice displayed significantly increased survival compared to controls (Figure 3.6C). Although spontaneous bleeding was not observed, hematocrit was modestly yet significantly reduced in untreated DKO mice (Figure 3.6D). Thus platelet glucose metabolism is essential for in vivo thrombosis.

Discussion

Several in vitro studies have suggested a relationship between glucose metabolism and platelet function (6, 7, 21), however, the specific role of glucose transport and glucose utilization to platelet function in vitro and in vivo was incompletely understood. For the first time our studies demonstrate the critical contributions of platelet glucose metabolism to platelet circulating half-life, activation, degranulation, and thrombus formation.

RNASeq analyses of platelets from mice and humans indicate that GLUT1 and GLUT3 are the only Class I glucose transporters expressed in platelets (22-24). Surprisingly, deletion of GLUT1 alone led to no change in glucose uptake in the presence or absence of thrombin, indicating that GLUT1 is not essential for glucose uptake, and that GLUT3 may effectively compensate for the loss of GLUT1. Possible adaptations include increased plasma membrane GLUT3 localization or an increase in GLUT3 activity. Deletion of GLUT3 alone only slightly decreases basal glucose uptake. However, GLUT3-KO platelets did not increase glucose uptake upon activation. Therefore GLUT3 translocation to the plasma membrane is largely responsible for activation-mediated increased glucose uptake in platelets. Individually GLUT1 and GLUT3 may be partially dispensable for platelet function, but loss of both transporters confirms the central role of platelet glucose utilization for normal physiological platelet function.

DKO mice developed thrombocytopenia. Studies in megakaryocytes revealed normal abundance and morphology, excluding an effect on megakaryocyte maturation and development.

However, the ability of megakaryocytes to generate platelets in response to platelet depletion was reduced, raising the possibility that platelet budding from megakaryocytes is a glucose-dependent process. Alternatively, the decrease in regeneration time may reflect increased clearance of nascent platelets, which is supported in part by decreased circulating half-life of DKO platelets. Although we could not identify any gross deficit in megakaryocyte function, we cannot completely rule out the possibility that megakaryocyte function is not altered in DKO mice.

In the absence of glucose metabolism, platelets increase mitochondrial respiration and mitochondrial membrane potential. This increase was not associated with increased mitochondrial content, indicating a qualitative rather than a quantitative mitochondrial adaptation. Metabolic plasticity in platelets has been previously observed in vitro following administration of fatty acids, glutamate, or pyruvate (25). Here we have shown that mitochondrial metabolism in the absence of glycolysis is insufficient to maintain physiological platelet function in vivo. Our studies in which alternate substrates were presented to DKO platelets in vitro revealed a partial reversal of platelet apoptosis. These observations identify an important role for mitochondrial metabolism in maintaining platelet viability.

When incubated in vitro, DKO platelets spontaneously expose PS. This exposure was rescued by the addition of mitochondrial substrates glutamate and pyruvate, indicating an energy-dependent mechanism. This PS exposure was accompanied by the loss of mitochondrial potential and increased caspase 3/7 activity. In addition, DKO platelets did not demonstrate altered sensitivity to the BH3 mimetic ABT-737, suggesting that DKO-mediated apoptosis is not facilitated by inherent alterations in BCL-XL activity. Because this apoptosis was partially inhibited by mitochondrial substrates, it is highly likely that platelet metabolism regulated platelet apoptosis and is responsible for the decrease in platelet circulating half-life. The possibility that metabolism can regulate platelet lifespan is not new (26), but these studies are the first to demonstrate a direct link.

In contrast to the increase in annexin v binding under basal conditions, DKO platelets

stimulated with thrombin and thrombin plus convulxin were unable to further increase PS exposure through the TMEM16F pathway. Unlike the caspase-3/7-dependent pathway, this PS exposure was facilitated by decreased calcium flux following administration of thrombin plus convulxin. It is possible that DKO platelets are unable to increase cytoplasmic calcium flux following stimulation due to a decreased ability to generate ATP for sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity. In contrast, when treated with the calcium ionophore A23187 with availability of extracellular calcium, DKO platelets did not manifest any defects in annexin v binding. Taken together, these data indicate that the deficiency in thrombin plus convulxin-mediated annexin v binding is due to decreased calcium flux, and signaling downstream of calcium flux does not require glucose metabolism. These data establish a link between metabolic function, calcium homeostasis, and platelet PS exposure that could contribute to in vivo thrombosis and complement system activation (27).

Glucose metabolism is essential for platelet activation. Previous studies of platelets in vitro have investigated the role of glucose metabolism to platelet function using 2-DOG to inhibit glucose uptake. These studies have indicated that glucose uptake and metabolism play a significant role in platelet activation (28). Our data are in agreement with these previous studies and further emphasize the fundamental importance of glucose metabolism to platelet function. Additionally, when calcium signaling is restored by administration of a calcium ionophore, GPIIbIIIa activation and α-granule release remain impaired, indicating that glucose metabolism is essential for platelet activation downstream of calcium signaling. Furthermore, here we show for the first time that glucose metabolism is essential for regulation of calcium flux and activation downstream of calcium signaling. In vivo DKO mice display increased bleeding following tail clip, increased time to occlusion in a model of arterial thrombosis, and increased survival in a pulmonary embolism model. These data demonstrate that in vivo, even in the presence of alternative metabolic substrates, glucose remains the essential substrate for platelet function.

In conclusion, these studies reveal an essential role for glucose metabolism in regulating

platelet activation and half-life. Elucidating the fundamental roles of glucose uptake and metabolism in platelet function provides the conceptual framework to better understand how the extracellular milieu could potentially alter platelet function in metabolic disorders such as diabetes.

Methods

Animals

All animal studies were approved by the institutional animal care and use committee (IACUC) of the University of Iowa and the University of Utah. Mice on a C57BL/6 background were housed under standard conditions of temperature and lighting. Pf4 Cre transgenic mice were obtained from Jackson laboratories on a C57B/6 background. GLUT3 mice were generated on a C57B/6 background, and GLUT1 mice were generated on a C57B/6 background as previously described (29). Experiments were conducted on male mice between 8 to 14 weeks old.

Platelet isolations

Whole blood was isolated from isoflurane-anesthetized mice via carotid artery cannulation into 1:20 acid-citrate-dextrose (ACD). Whole blood was then diluted with pipes saline glucose (PSG) buffer and centrifuged at 120g for 10 minutes. Platelet-rich plasma was then diluted with PSG with prostaglandin E1 PGE_1 (10nM) and centrifuged 378g x 10min and centrifuged again at 378g x 10 minutes. When noted, platelets were incubated with Ter119- and CD45-labeled microbeads (Miltenyi Biotec, Auburn CA) and negatively depleted of red blood cells and leukocytes. Following the isolation and centrifugation steps, platelets were allowed to rest for 30 minutes prior to experimental manipulation. Platelet counts were determined by Cellometer Auto M10 (Nexcelom Bioscience, Lawrence, MA).

Glucose uptake

Washed platelets at a concentration of $2x10^8$/mL in DMEM with 1mM glucose were incubated with 10mM H^3-2-Deoxy-D-Glucose in the presence or absence of 1U/mL thrombin or 10μM Cytochalasin B. Samples were incubated for 10 minutes, washed 3 times in Stop Buffer (Ca^{2+} Free HBSS with 10μM Cytochalasin B), and solubilized in 1M NaOH. BCA protein analysis was conducted in parallel aliquots.

Seahorse flux analysis

Seahorse analysis were conducted as previously described (30), with the addition of leukocyte and red blood cell depletion using Terr119 and CD45 micro beads. Platelets were seeded at a density of $1x10^8$ platelets/well in Seahorse XF24 Analyzer plates (Seahorse Bioscience, North Billerica, MA) treated with or without 1U/mL thrombin. Data normalized to platelet counts.

Whole blood analysis

Platelet counts and hematocrit was determined via laser-based Advia 120 whole-blood analyzer (Siemens, Germany). Blood was collected by cheek bleed into EDTA-coated capillaries, diluted 1:10 in saline, and analyzed.

Platelet regeneration assay

Baseline platelet counts were determined by flow cytometry counting of diluted whole blood, gated for CD41-APC-positive events and normalized to flow beads (BD Bioscience, San Jose, CA). DKO and littermate control mice were injected i.v. with 2μg/g anti-GP1bα antibody (Emfret Analytics, Germany). Platelet counts were obtained every 24 hours for 168 hours.

Megakaryocyte cultures

Briefly, bone marrow from DKO or littermate control mice was flushed, and filtered through 100μM cell filters. Bone marrow was then cultured in DMEM with 5mM glucose,

glutamate, and recombinant thrombopoietin (TPO) for 5 days. At day 6, megakaryocytes were enriched using a gravity BSA gradient 1.5 to 3%. Megakaryocytes were then cultured overnight on fibrinogen coated chamber slides for experiments. Megakaryocytes producing platelets were quantified and normalized to total megakaryocytes.

Bone marrow megakaryocyte density

Femurs isolated from DKO and littermate control mice were fixed in 2% PFA overnight. Bones were then decalcified in 10% HCl for 2 hours, then incubated in 14% EDTA for 3 days, and embedded in paraffin, followed by antigen retrieval at 95^{o}C in citrate buffer. Slides were then incubated with anti-Vwf antibody and counterstained with haematoxylin. Femurs were imaged at 40x on a Leica DM6000B slide-scanning microscope. Megakaryocytes were counted and categorized as ≤30μm diameter or >30μm diameter per μm^2 bone marrow area, with 5 frames per femur.

Circulating half-life

Mice were injected with Anti-GP1bβ-FITC antibody (Emfret Analytics, Germany) and blood was obtained every 24 hours via cheek. Platelet counts were determined as percent GP1bβ-FITC positive CD41-APC events in diluted whole blood. Mice were monitored for 120 hours.

Apoptosis studies

Washed platelets were incubated in DMEM supplemented with 5mM glucose and, when noted, 1mM sodium pyruvate and 2mM glutamate. Platelets analyzed longer than 1 hour after incubation were kept at 37^{o}C with 5% CO_2 until analysis. Caspase 3/7 activity was determined using Apo-ONE Homogeneous Caspase 3/7 assay (Promega, Madison WI). Thrombin + convulxin-mediated annexin V activation was induced using 1U/mL human thrombin and 400ng/mL convulxin and incubated for 15 minutes. Mitochondrial potential was determined using flow cytometry. CD41-APC positive washed platelets were gated and analyzed for TMRM

geo. MFI, following analysis, platelets were treated with 100nM carbonyl cyanide m-chlorophenyl hydrazine (CCCP) for 10 minutes then analyzed once more. Mitochondrial potential was determined as basal TMRM Geo. MFI minus (-) CCCP treated TMRM Geo. MFI. Abt-737 was incubated with washed platelets in 5mM glucose DMEM for 2 hours at 37^{o}C with 5% CO_2. Caspase 3/7 activity (Promega, Madison, WI) was assessed. In addition, annexin v positivity was determined using flow cytometry.

Platelet calcium content

Calcium content of CD41-APC-positive washed platelets in DMEM with specified substrates was analyzed by flow cytometry. Platelets were loaded with 5μM Fluo-4 for 30 minutes at 37^{o}C with 5% CO_2. Platelets were then diluted 1:10 in 1mM EGTA HBSS without Ca^{2+}/Mg^{2+} and immediately analyzed. Diluted platelets were then treated with 100nM Ionomycin and analyzed. Data were normalized to average of control value, unstimulated values of samples on the given day, and specified time point of experiment.

Whole blood platelet activation

Fresh whole blood was diluted 1:10 in Ca^{2+}-free HT buffer. Diluted blood was then added to the specified agonist in the presence of JonA-PE, CD62p-FITC, (Emfret Analytics, Germany) and CD41-APC (ebioscience, San Diego, CA) antibodies, to a final Ca^{2+} concentration of 5mM. Samples were then incubated at 37^{o} C for 10 minutes and fixed with 5 volumes of FACs lysis buffer (Beckman Dickson, San Jose, CA). Samples were then analyzed using flow cytometry LSR II (Beckman Dickson, San Jose, CA) gating for CD41 positive events.

Transmission electron microscopy

Washed platelets were incubated in DMEM in the presence or absence of 250uM Par4 peptide for 10 minutes at room temperature. Platelet stimulation was terminated by the addition of equal volumes 4% gluteraldehyde. Following 30-minute gluteraldehyde incubation, platelets

were gently centrifuged at 1500rpm for 10 minutes, and resuspended in 4% glutaraldehyde. Samples were then processed as previously described for electron microscopy.

In vivo thrombosis

Ferric-chloride-induced arterial thrombosis: mice were anesthetized with pentobarbital and mechanically ventilated. Exposed right carotid arteries were then treated with 1mm x1mm whatman filter paper saturated with 7.5% ferric chloride for 3 minutes, rinsed with saline, and flow rate was monitored with a 0.5 PSB Doppler flow probe (transonic Systems Inc, Ithaca, NY). Time to occlusion was monitored (31). Tail bleed analysis was conducted on mice anesthetized with 2.5% isoflurane. Tails were excised 3mm from the tip and submerged in warm saline, time to bleeding cessation for 10 seconds was monitored. Collagen/epinephrine-induced pulmonary embolism: mice were injected i.v. with a mixture of 20 μ g/Kg epinephrine (Hospira Inc., Lake Forest, IL), 430 μ g/Kg collagen (Chrono-Log, Columbia, MD) in PBS. Time of survival was determined by monitoring chest palpations, and time of death was determined as time when breathing ceased and did not return for 1 minute.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6, or Microsoft Office Excel 2011. Add data presented as mean ±SEM. Statistical significance threshold of $p<0.05$ was utilized.

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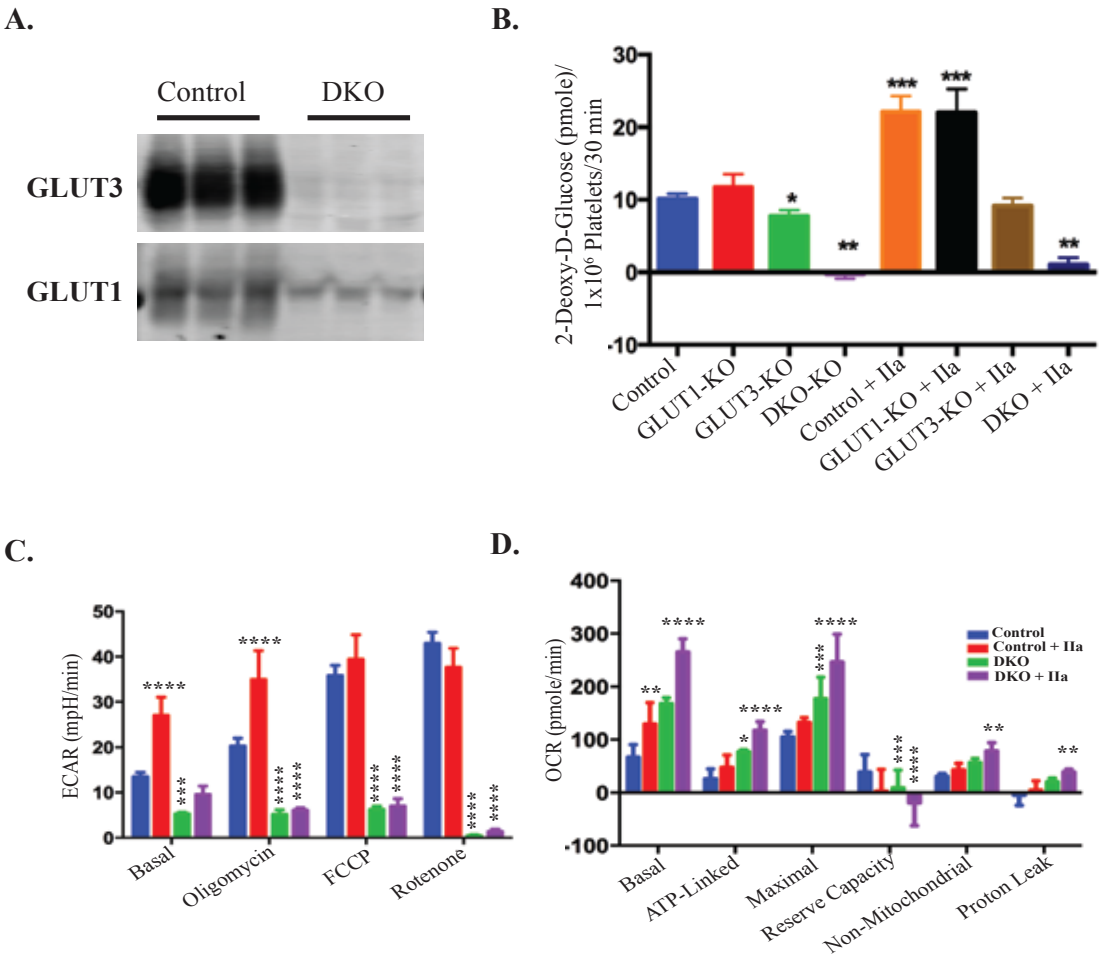

Figure 3.1. Glucose metabolism and mitochondrial O_2 consumption in GLUT1 KO, GLUT3 KO and DKO platelets. A. Western blot analysis of GLUT1 and GLUT3 expression in DKO and littermate control platelets, n=6. B. [H^3]-2-Deoxy-D-glucose uptake in washed platelet under basal and thrombin (1U/mL) stimulated conditions, n=14, DKO/control n=3. C. Seahorse analysis of CD45- and Ter119-depleted platelets, monitoring extracellular acidification rate, n=3. D. Oxygen consumption rates of CD45- and Terr119-depleted platelets using the oxygen consumption rate, determined using the Seahorse XF24 analyzer, n=3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; (2-way ANOVA followed by Tukey's multiple comparison post hoc test.

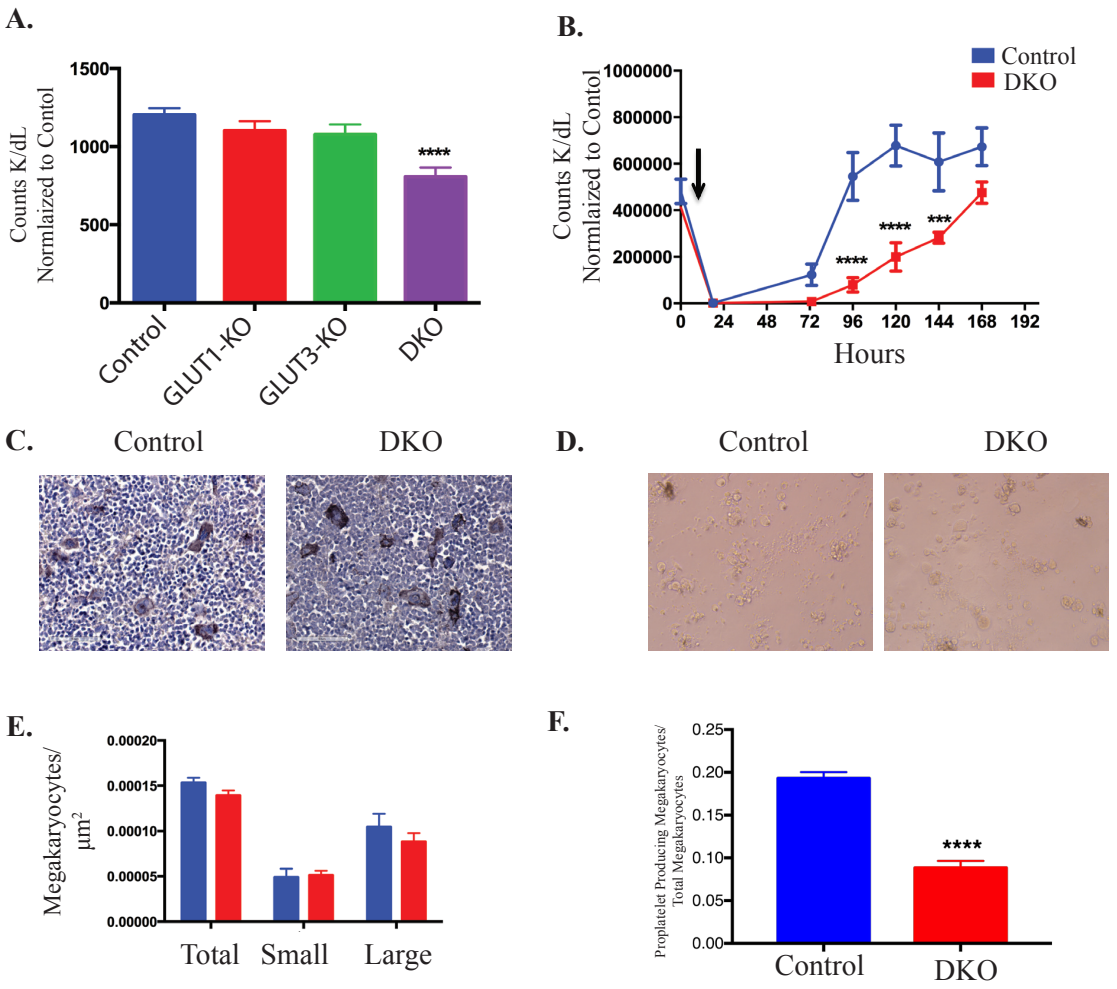

Figure 3.2. Thrombocytopenia and decreased platelet regeneration in DKO mice. A. Platelet counts in whole blood, n=7. B. Mice were injected with anti-GPIbα antibody (arrow) and platelet counts were monitored serially using flow cytometry, n=6. C. Representative cross-sectional images of femurs using IHC analysis of Vwf as a marker of megakaryocytes, and counterstained with haematoxylin. D. Quantification of megakaryocyte number per μm^2 of bone marrow area, n=3. E. Representative images of megakaryocytes isolated from femurs and tibias of control of DKO mice, which were isolated and cultured in thrombopoietin-enriched DMEM for 5 days, separated on a BSA density gradient, and cultured on fibrinogen overnight. F. Quantification of ratio of total megakaryocytes vs. proportion with pro-platelet forming extensions, n=6. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; (2-way ANOVA followed by Tukey's multiple comparison post hoc test.

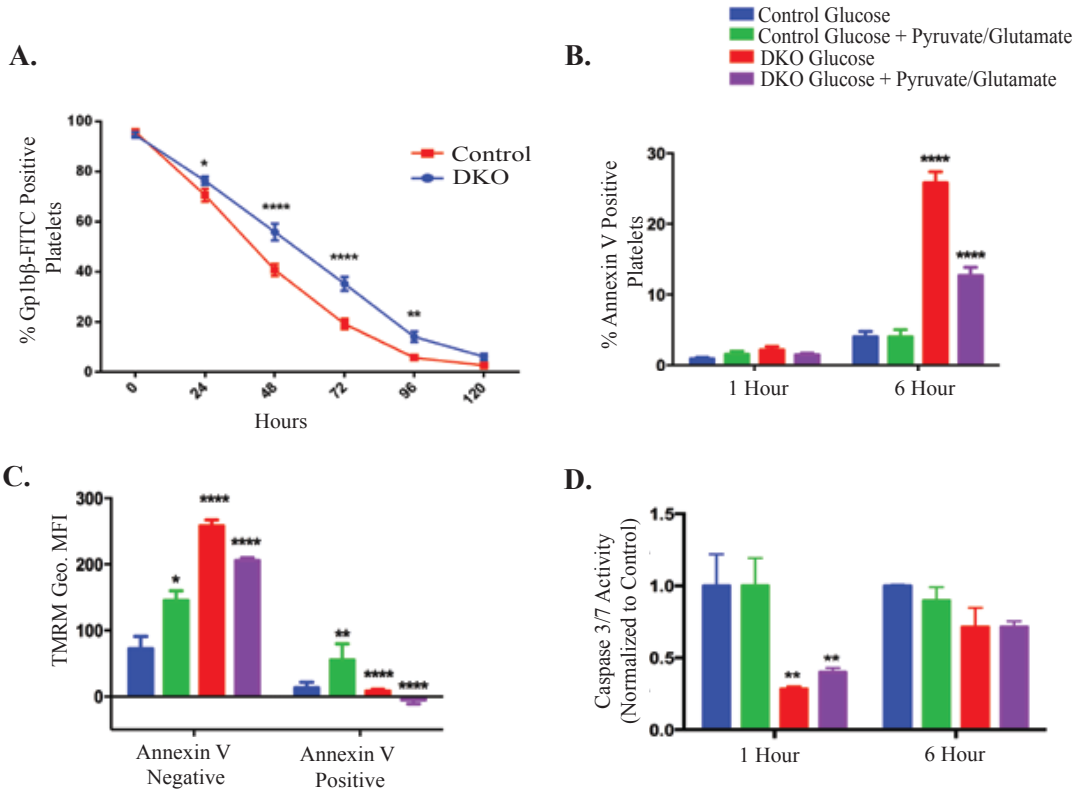

Figure 3.3. DKO platelets expose phosphatidylserine spontaneously in response to metabolic stress. A. Mice injected with dylight-488-conjugated GPIbβ antibody were monitored for GPIbβ-488 positive events, n=6. B. Platelets were cultured in 5mM glucose DMEM with or without 2mM glutamic acid and 1mM pyruvate for 6 hours, and then analyzed for annexin V-FITC positive events (n=14) and C.) TMRM Geo. MFI., n=3 D. Caspase 3/7 activity of platelets incubated for 1 and 6 hours in 5mM glucose ±glutamate and pyruvate, n=5. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; (2-way ANOVA followed by Tukey's multiple comparison post hoc test.

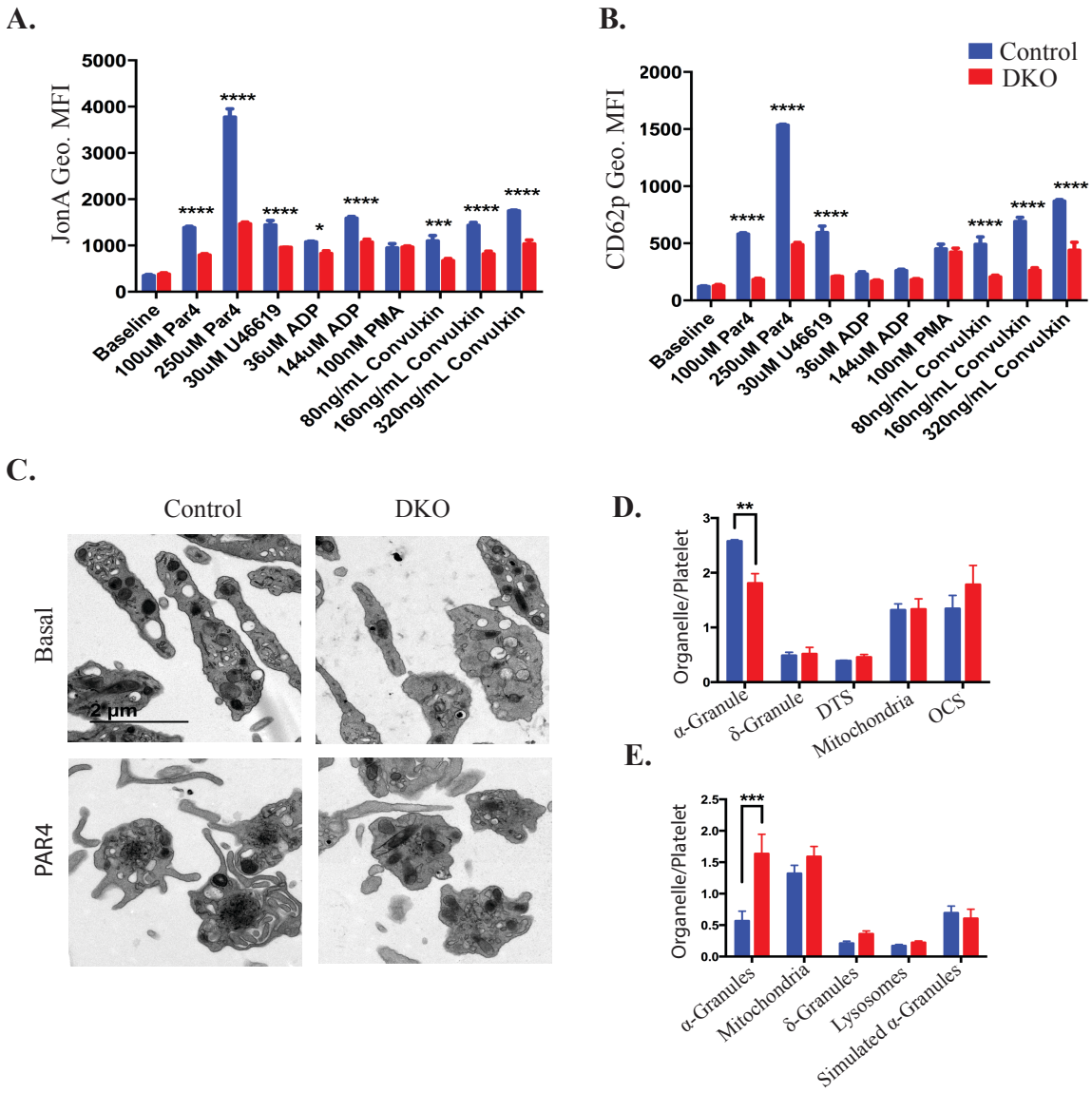

Figure 3.4. DKO platelets have decreased activation in vitro. Diluted whole blood treated with multiple concentrations of the indicated agonists was gated for CD41-APC-positive events. A. GPIIbIIIa complex activation marked by JonA-PE binding B. CD62p-FITC surface translocation represented as the geometric mean fluorescence (n=3). C. Platelets incubated for 1 hour in 5mM glucose DMEM were then stimulated for 15 minutes with thrombin 1U/mL, thrombin 1U/mL plus Convulxin 400ng/mL, or 10µM A2387 and analyzed for annexin V binding, n=3. D. Thrombin plus convulxin mediated activation of GPIIbIIIa and E). CD62p surface translocation, n=3. F. Platelets loaded with Fluo-4 were stimulated with thrombin and convulxin Geo. MFI was monitored pre- and postactivation, n=3. G. Transmission electron micrographs of platelets in DMEM in the presence or absence of 250µM PAR4 peptide. H-I. Quantification of ultrastructural characteristics based on 10 fields per mouse, n=4. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; (2-way ANOVA followed by Tukey's multiple comparison post hoc test.

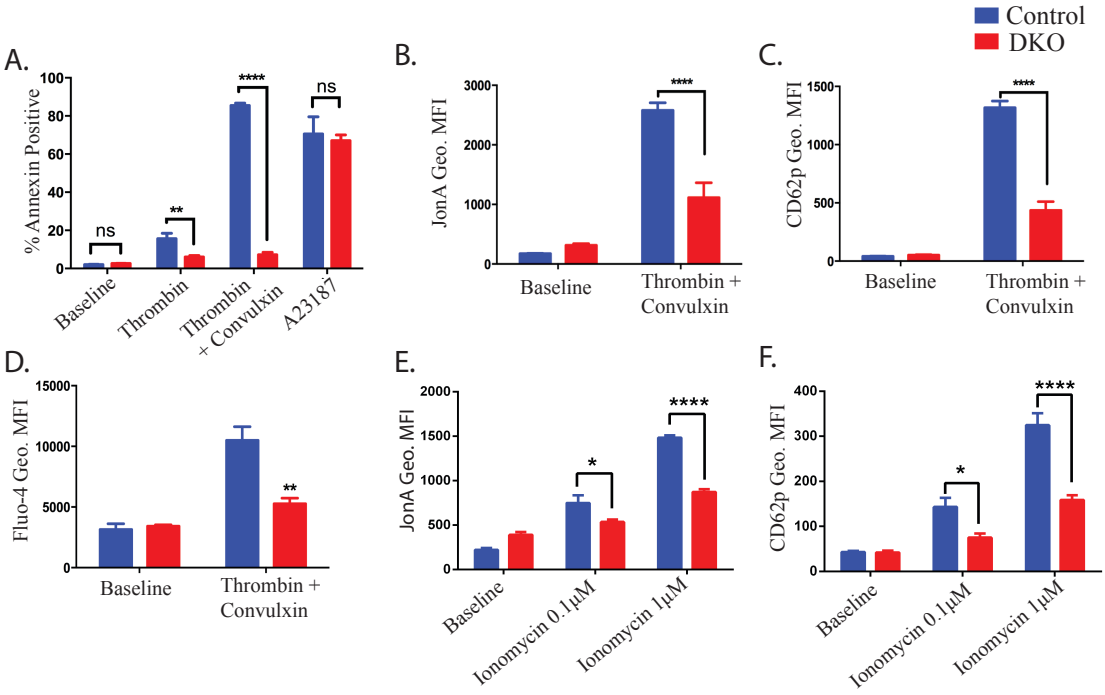

Figure 3.5. Calcium signaling is impaired in DKO platelets. A. Percent annexin-v-positive platelets were monitored following stimulation with thrombin 1U/mL, thrombin 1U/mL + convulxin 360ng/mL, or 10μM A23187, n=6. B. Thrombin 1U/mL plus convulxin 360ng/mL was administered and JonA binding B. CD62p binding C., n=3. Calcium flux was monitored following similar stimulation by Fluo-4 Geo. MFI D. n=3. The ability of calcium ionophore ionomycin to induce binding to induced JonA E. and CD62p F. binding was monitored, n=3. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; (2-way ANOVA followed by Tukey's multiple comparison post hoc test.

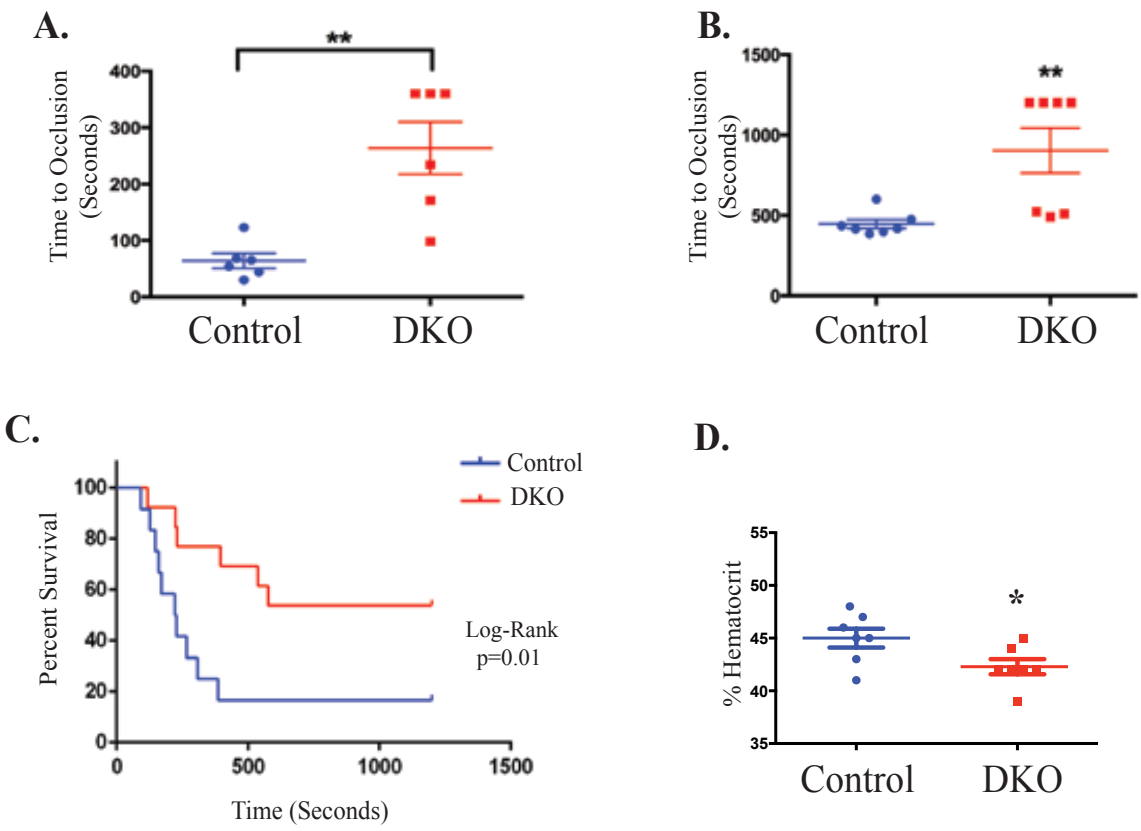

Figure 3.6. DKO mice display decreased thrombosis in vivo. A. DKO mice subjected to tail bleeding displayed prolonged time to cessation, n=6. B. Mice were subjected to 7.5% $FeCl_3$-induced arterial thrombosis and time to occlusion was monitored using a doppler flow probe, n=7. C. Collagen/Epinephrine-induced pulmonary embolism, n=12. D. Hematocrit analysis as determined via ADVIA 120, n=7. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; students two-tailed t-test (A, B, AND D); Log-rank (Mantel-Cox) test (C).

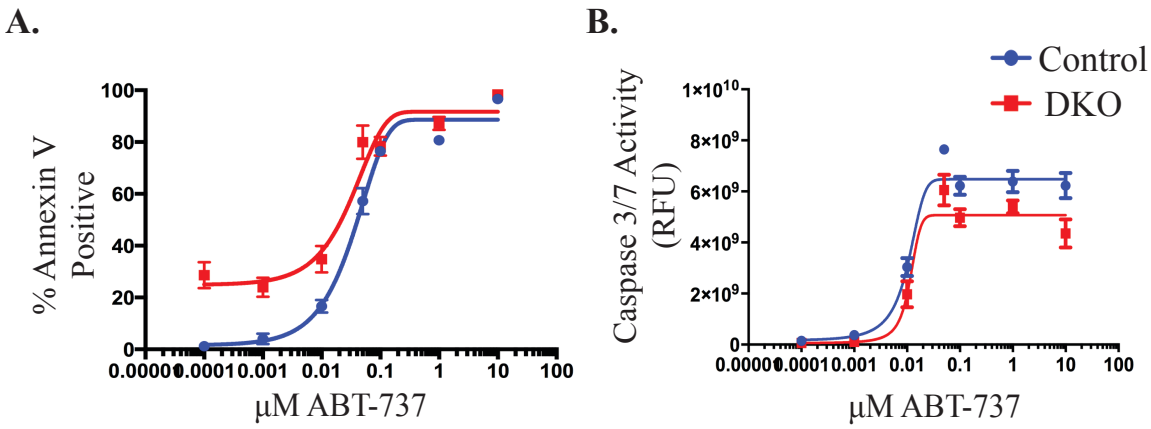

Figure 3.7. ABT-737 dose response. Platelets incubated for 2 hours with the indicated concentrations of ABT-737 in 5mM glucose DMEM and monitored in parallel for percent annexin V-FITC positive platelets, n=6 (A) and caspase 3/7 activity, n=3 (B).

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Abstract

Upon activation, platelets increase glucose uptake, glucose oxidation, glycogen utilization, and lactic acid production. Although this dramatic increase in metabolism has been well characterized, the mechanisms regulating glucose metabolism in platelets are not well understood. Recent analyses of mice lacking glucose transporters 1 & 3 have directly linked glucose metabolism to platelet function in vitro and in vivo. These studies have also demonstrated a multifaceted requirement of glucose metabolism for platelet activation, and suggest that alterations in the metabolic milieu can lead to changes in platelet function. These data are particularly of interest because patients with diabetes have increased platelet activation and thrombosis. Together these new insights demonstrate a need to better understand how the extracellular milieu can regulate glucose metabolism, and how this may lead to dysfunctional platelets in disease states like diabetes.

Introduction

In platelets, metabolism-derived cycling of ATP is highly dynamic, occurring at a rate similar to neutrophils and leukocytes and 150-times higher than that in erythrocytes (1-3). In addition, platelet activation and clot formation lead to rapid energy production and utilization, as demonstrated by increased glucose uptake (4), glucose oxidation (5), glycogen utilization (6), lactic acid production (7), and mitochondrial respiration (8). This correlation between glucose metabolism and platelet function has been recognized for nearly half a century; however, few

studies have investigated the mechanisms by which glucose metabolism contributes to platelet function. Recent studies of mice lacking glucose transporters specifically in platelets have demonstrated that glucose metabolism can regulate calcium signaling, degranulation, activation of glycoprotein IIbIIIa (GPIIbIIIa), exposure of phosphatidylserine (PS) to the outer leaflet of the plasma membrane, platelet circulating half-life, and in vivo thrombosis (Chapter 3). These studies establish that decreased glucose metabolism results in platelet dysfunction through multiple mechanisms, and raise questions that are integral to platelet biology.

Platelet metabolism can be regulated by the extracellular milieu. In vivo and in vitro hyperglycemic conditions can lead to increased platelet glucose metabolism (9), activation, and thrombosis (10). In vitro studies of platelets using inhibitors of glucose uptake and metabolism have demonstrated that glucose utilization is essential for platelet activation (11, 12). In the absence of glucose, platelets display a marked metabolic plasticity, resulting in increased utilization of alternative mitochondrial substrates (Chapter 2). This plasticity may be of particular importance in disease states such as type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) because the composition of the metabolic milieu is altered. The consequence of alternate substrate utilization in diabetes is unknown, but diabetic patients exhibit increased platelet activation and thrombosis (13). Because recent studies have directly linked glucose metabolism to platelet function in vivo; examination of the metabolic mechanisms that result in platelet dysfunction is warranted.

Glucose transporters in platelets

Glucose transporters mediate glucose uptake into cells through facilitated diffusion (14). RNA and proteomic analysis of murine and human platelets indicates that glucose transporter 1 (GLUT1) and glucose transporter 3 (GLUT3) are the only glucose transporters present (15, 16) (Chapter 3). Recent investigations of mice lacking GLUT1 and GLUT3 specifically in platelets demonstrate that these transporters maintain redundant functions while also exhibiting unique

functions based on their localization (Chapter 2) (Figure 4.1). In addition, these studies found that GLUT1 and GLUT3 are the sole glucose-transporting proteins in platelets.

Glucose Transporter 1

When GLUT1 was specifically knocked out of platelets, we observed no major alteration in glucose metabolism. Surprisingly, glucose uptake, basal glycolytic rates, and glycolytic intermediates were unchanged in platelets from GLUT1-KO mice. However, the pentose phosphate pathway (PPP) intermediate sedoheptulose-7-phosphate was significantly decreased and other PPP intermediates trended downward (data not shown). Although no major alteration in glucose metabolism was detected in these mice, functional analysis indicates that these mice have increased platelet activation and thrombosis. Currently, the contribution of GLUT1-mediated metabolism to platelet function is unknown.

RNASeq analysis of cultured murine megakaryocytes indicates that GLUT1 transcript is present, but very little mRNA was found in platelets. Additionally, western blot analysis of GLUT1 in megakaryocytes and platelets indicates that GLUT1 protein is present in both cell types (Chapter 3) (4, 15). These data suggest that GLUT1 protein is translated in the megakaryocyte and packed into platelets.

GLUT1 imports glucose into platelets and may account for ~80% of basal glucose uptake. This was determined through analysis of platelets lacking GLUT3, where basal glucose uptake decreased by 20% and GLUT1 was the sole glucose transporter remaining. Surprisingly, GLUT1-deficient platelets display no alteration in glucose uptake, presumably due to a post-translational compensatory effect of GLUT3. These studies demonstrated that GLUT1 and GLUT3 exhibition redundant functions, and underscores the importance of glucose metabolism to platelet function.

The generation of GLUT1-deficient platelets perhaps raised more questions than it answered. Investigations using immunohistochemistry have failed to accurately determine the

localization of GLUT1 in platelets. Moreover, because GLUT1 is translated in megakaryocytes it may be responsible for glucose uptake into megakaryocytes. Perhaps disruption of this process may lead to the production of dysfunctional platelets, which are observed in the GLUT1-KO mice. Although the exact contribution of GLUT1 to platelet function remains unclear, these studies demonstrated that GLUT1 does transport glucose into platelets, and that deletion of GLUT1 leads to significant defects in platelet function.

Glucose Transporter 3

GLUT3 mRNA is highly expressed in humans and murine platelets (17); in fact, GLUT3 mRNA is ranked in the top 94 percentile (18) of all transcripts in human platelets. In addition, mouse megakaryocytes (Chapter 2) and the immortalized human megakaryocyte cell lines MEG-01, DAMI, and CHRF all revel the presence of GLUT3 mRNA (4). Interestingly, in humans, black individuals have decreased GLUT3 mRNA expression compared to whites (18).

GLUT3 function is regulated by its subcellular localization (Chapter 2). Immunogold labeling of GLUT3 in resting platelets indicates that 85% of GLUT3 is expressed in α-granule membranes and roughly 15% is expressed in the plasma membrane. Under basal conditions, GLUT3 was responsible for at least ~20% of glucose uptake into platelets. However, upon stimulation, platelets degranulate and the α-granule membranes fuse with the plasma membrane, leading to GLUT3 incorporation into the plasma membrane (19). This incorporation of GLUT3 into the plasma membrane leads to a 2-fold increase in glucose uptake (Chapter 2). In addition to assisting cytoplasmic glucose uptake, GLUT3 can facilitate glucose uptake into α-granules for intragranular glycolysis (Chapter 2). These data demonstrate that the function of GLUT3 is regulated by its subcellular localization.

In platelets, GLUT3 facilitates at least 3 distinct functions. First, GLUT3 mediates at least 20% of basal glucose uptake into platelets. Second, GLUT3 is responsible for the 2-fold increase in glucose uptake following platelet activation. Third, GLUT3 mediates glucose entry

into α-granules for intragranular glycolysis. Studies of mice with a platelet-specific knockout of GLUT3 have demonstrated that loss of GLUT3 leads to decreased platelet function in vitro and in vivo. The contribution of this GLUT3-mediated transport to platelet function will be discussed in detail in this review.

Glucose metabolism in platelets

Once glucose enters the platelet, it can be utilized for multiple functions. Platelets contain all of the enzymes required for metabolizing glucose through glycolysis, the pentose phosphate pathway, and polyol pathway, with various implications for cellular function.

Glycolysis

Under resting conditions, washed platelets utilize roughly 55% of imported glucose for glycolysis to be excreted as lactic acid (20), with an additional ~16% undergoing glycolytic conversion to pyruvate and utilized for oxidative phosphorylation (5). Platelets maintain a highly dynamic cycling of ATP (1-3), but the exact reason for this rapid energy cycling under basal conditions is unknown. Unlike nucleated cells, once platelets are circulating, they exhibit only minimal protein translation (21). Recent reports, however, demonstrated that platelets contain active proteasomes, whose activity is ATP-dependent and essential for platelet function (22). Another reason why platelets exhibit rapid energy cycling is that basal energy maintenance is required to prime platelets for rapid activation (23). Recently, calcium signaling was shown to be disrupted in platelets lacking glucose uptake, thereby suggesting a third possibility that glucose metabolism is required for calcium cycling, possibly by supplying ATP to sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and/or the plasma membrane Ca^{2+}-ATPase (PMCA). Clarifying what the requirement of glucose metabolism for basal platelet function is may advance our understanding of how platelets respond to altered metabolic states.

α-Granule-mediated glycolysis

Under basal conditions, GLUT3 facilitates glucose uptake into α-granules for glycolysis (Chapter 2). This uptake was determined in a saponin-permeabilized model, where α-granules in the absence of cytosol were capable of metabolizing C^{13}-glucose into C^{13}-lactic acid in a GLUT3-dependent manner. These data indicate that GLUT3 facilitates glucose uptake into α-granules for glycolysis.

GLUT3-KO platelets, which lack the ability to facilitate intragranular glycolysis, display decreased α-granule degranulation and cargo release, as well as decreased in vivo thrombosis. Permeabilized platelets, devoid of cytosol but with intact α-granules, can be stimulated by calcium to release the α-granule contents. Importantly, this process cannot occur without the addition of ATP, indicating that ATP is required for platelet degranulation. It is possible that intragranular glycolysis generates the ATP required for α-granule degranulation. This newly exposed mechanism of platelet glucose metabolism raises many questions. Why do platelets need to compartmentalize glycolysis? Maybe platelets require ATP in the lumen of α-granules to fuel ATP-dependent proteins that are required for degranulation. For example, it is possible that ATP derived from glycolysis is utilized by N-ethylmaleimide-sensitive factor (NSF), an ATPase essential for α-granule release. In addition to NSF, multiple ATP-dependent proteins have been identified through α-granule proteomic analyses (24). Clearly, intragranular glycolysis would yield ATP in the α-granule lumen, making it readily available to proteins requiring ATP, which would likely be found in the lumen as well. These new findings suggest a unique mechanism by which platelets degranulate.

Glucose metabolism is essential for platelet activation

Thrombin-stimulated platelets display a nearly 2-fold increase in lactic acid production (7) and glucose oxidation (5). The contribution of glucose metabolism at the time of activation has recently been investigated. Following activation, there is a rapid increase in glucose

metabolism that is thought to fuel the estimated 3-fold increase in ATP utilization, occurring within the first 60 seconds of activation (1, 3). Upon activation, platelets increase actin polymerization, leading to dramatic cytoskeletal rearrangement, protein synthesis, granule release, and integrin complex activation, all of which require substantial amounts of energy. The contribution of glucose metabolism to these functions will be discussed below.

Various studies have investigated the contribution of glucose metabolism to platelet function in vitro. To do this, investigators have treated platelets with modified glucose concentrations or administered 2-deoxyglucose (2-DOG). 2-DOG is a glucose analog that competitively inhibits glucose uptake into platelets, and accumulates in the cytoplasm in the phosphorylated form, ultimately leading to toxicity (11). Platelets incubated in the absence of glucose or presence of 2-DOG have greater than 50% decrease in aggregation (23). Platelets pretreated with 2-DOG and the mitochondrial respiration inhibitor antimycin A show complete inability to activate in response to thrombin (12); however, antimycin A alone does not affect platelet aggregation. This phenomenon reflects the important ability of platelets to utilize different substrates and metabolic pathways to produce ATP. Recently, studies of platelets lacking both GLUT1 and GLUT3 (DKO) have demonstrated that platelet glucose metabolism is essential for calcium signaling, degranulation, and GPIIbIIIa complex activation. Studies of DKO platelets have shown direct mechanisms by which glucose metabolism can regulate platelet function.

Alteration in calcium flux appears to be one such mechanism. Specifically, DKO platelets fail to increase cytoplasmic calcium flux following stimulation in vitro. Agonist-mediated signal transduction converges on increasing calcium concentrations in the cytoplasm through the release of stored calcium and influx of calcium from the extracellular environment. When DKO platelets were stimulated with convulxin plus thrombin at high concentrations, they failed to increase cytoplasmic calcium concentrations. It is unclear whether this decrease in calcium flux is due to decreased signal transduction, decreased calcium stores, or an impaired

ability to import calcium following stimulation.

Agonist-mediated calcium release into the cytoplasm is regulated by PLCβ- and PLCγ-mediated production of inositol triphosphate (IP3) and diacylglycerol (DAG). While thrombin leads to the activation of PLCβ, convulxin results in PLCγ activation. Therefore, in the presence of both convulxin plus thrombin both PLC isotypes become activated. Following this co-stimulation, DKO platelets were still unable to increase cytoplasmic calcium flux; therefore, it is unlikely that DKO platelets have an inherent impairment in both PLC proteins. Yet it is also possible that IP3 and DAG formation is impaired. IP3 generation leads to the activation of IP3 receptors in the dense tubule system (DTS), where calcium stores are located in platelets. Once activated, IP3 receptors release stored calcium into the cytosol. Although IP3-induced release of stored calcium is not ATP-dependent, the maintenance of this calcium pool is dependent on the calcium ATPase SERCA. It is thus possible that SERCA activity is decreased in the absence of glucose metabolism, leading to the loss of stored calcium so when IP3R are activated, there is no stored calcium to release. Maintenance of calcium stores is facilitated by stromal interaction molecule 1 (STIM1)-coupled regulation of calcium release-activated calcium channel protein 1 (Orai1), which mediates calcium influx from the extracellular environment. It is possible that a compensatory increase in cytoplasmic calcium occurs, due to increased Orai1 activation, because calcium stores are compromised in DKO platelets. In addition, calcium can enter the cytosol from the extracellular environment through activation of TRPC6 calcium cannels by DAG in an ATP-independent manner. Currently, the mechanisms by which glucose metabolism is required for calcium signaling in platelets remains unknown, and future studies will have to address how glucose metabolism and energy availability play a role in regulating calcium signaling.

A second mechanism through which glucose metabolism might regulate platelet function is by affecting the degranulation process. Agonist-stimulated DKO platelets display blunted activation of the GPIIbIIIa complex, degranulation, and exposure of PS to the outer leaflet of the plasma membrane. This finding was not surprising because calcium signaling, which is an

essential intermediate signaling event in these processes, was impaired. In order to determine if processes downstream of calcium signaling were also regulated by glucose metabolism, calcium signaling was restored in the DKO platelets by administration of a calcium ionophore. Ionophore treatment was able to restore PS exposure, but GPIIbIIIa activation and degranulation remained impaired. Together these data demonstrate that glucose metabolism is essential for multiple activated-dependent functions.

Agonist-mediated exposure of PS to the outer leaflet of the plasma membrane is impaired in DKO platelets. This exposure is facilitated by the calcium-sensitive scramblase TMEM16f. TMEM16f function is not well understood, but patients with Scott's syndrome have a mutation in this protein, which prevents agonist-mediated PS exposure. Unlike PS exposure through the platelet apoptosis pathway, discussed below, agonist-mediated PS exposure does not lead to the production of an "Eat Me" signal for phagocytic cells. Rather, agonist-mediated PS exposure is important for the regulation of thrombus formation by serving as a scaffold for coagulation proteins (25). Because agonist-mediated calcium signaling was decreased in DKO platelets and PS exposure is dependent on calcium signaling, platelets were treated with a calcium ionophore in vitro to restore calcium signaling. In the presence of a calcium ionophore, PS exposure to the outer leaflet of the plasma membrane was restored, indicating that processes downstream of calcium signaling do not require glucose metabolism. Together these data demonstrate that glucose metabolism is essential for PS exposure to the outer leaflet of the plasma membrane by aiding in calcium signaling, and that TMEM16f scramblase activity does not require on glucose metabolism.

DKO platelets exhibited a significant reduction in GPIIbIIIa activation in response to PAR, thromboxane, purinergic, and GPVI receptor agonists. Because agonist-mediated activation of the GPIIbIIIa complex is dependent on calcium signaling, and DKO platelets have decreased calcium signaling, platelets were treated with a calcium ionophore. Even in response to a calcium ionophore, DKO platelets had markedly reduced activation of the GPIIbIIIa complex,

indicating that glucose metabolism is essential for complex activation. This impaired activation may be caused by decreased ATP generation, which is required for cytoskeletal remodeling and anchoring of the GPIIbIIIa complex to the cytoskeleton. In order for GPIIbIIIa to move to its activated conformation, Arp2/3 is required to polymerize actin through an ATP-dependent manner. It is also possible that the decrease in GPIIbIIIa complex activation is due to the loss of DAG-mediated PKC activation, which can result in complex activation as well. Future studies are required to investigate the exact mechanisms by which glucose metabolism regulates activation of the GPIIbIIIa complex.

Glucose metabolism also is required for degranulation. This requirement was observed in platelets lacking intragranular glycolysis (Chapter 2), as well as in platelets in which glucose uptake was completely blunted (Chapter 3). Because DKO platelets failed to increase calcium signaling in response to multiple agonists, platelets were treated with a calcium ionophore. Even in the presence of an ionophore, DKO platelets displayed impaired degranulation, which may have been due to decreased intragranular glycolysis. These data are consistent with studies of permeabilized platelets, which are known to require ATP in order to degranulate.

Previous studies have investigated the contribution of ATP to platelet function. To do this, platelets were pretreated with 2-DOG and antimycin A for increasing lengths of time and then treated with thrombin. Platelet ATP, ADP, and AMP were simultaneously monitored to gain an estimation of the platelet energy state. Based on these experiments, it was estimated that the platelet energy requirements for activation responses could be ranked as follows: aggregation< dense granule and alpha granule secretion< acid hydrolase secretion< phosphatidylinositol breakdown, phosphatidate formation and arachidonate liberation (2). Although these studies were unable to account for sequential energy demands, the activation markers were treated independently of one another. These studies suggest that different platelet functions may be alternatively regulated by ATP demand, however the exact contribution of ATP production to platelet function requires further investigation.

GLUT3 facilitates glucose uptake following activation

Upon activation, GLUT3 translocates from α-granule membranes to the plasma membrane, and mediates a 2-fold increase in glucose uptake. The significance of this degranulation-mediated increase in platelet glucose uptake is unknown. Because the degranulation and subsequent translocation of GLUT3 is required for glucose uptake to occur, it is unlikely that activation-mediated glucose uptake supplies the increased energy demands for platelet activation. Therefore, the question arises: why do platelets increase glucose uptake following degranulation? Here we will discus what is known and the outstanding questions as to why platelets increase glucose uptake following activation.

Platelets lacking GLUT3 do not increase glucose uptake following stimulation, but activation-mediated glycolysis remains unchanged. Functionally, platelets from these mice exhibit blunted in vitro activation of GPIIbIIIa complex, α-granule degranulation and cargo release. In vivo, these mice demonstrated increased survival in a model of collagen/epinephrine-induced pulmonary embolism and decreased disease progression in a model of autoimmune inflammatory arthritis. Because basal and thrombin-stimulated glycolysis rates were unchanged in these mice, the contribution of GLUT3 to these functional defects were attributed to GLUT3-mediated α-granule glycolysis.

There are potentially multiple mechanisms by which platelets increase glucose uptake following stimulation. Human platelets contain ~40 α-granules per platelet, and although degranulation occurs rapidly, it is possible that platelets degranulate in a sequential manner. Consequently, platelets may have enough glucose energy or stored glycogen to facilitate the degranulation of an initial quantity of α-granules. Then the translocation of GLUT3 in these initial α-granules leads to increased glucose uptake, which fuels subsequent α-granule degranulation. This possibility is supported by the fact that platelets utilize glycogen upon stimulation, and GLUT3-deficient platelets do not completely lack degranulation; rather, degranulation is only somewhat blunted. However, this possibility is seemingly contradicted by

the fact that thrombin-mediated glycolysis is unchanged in GLUT3-KO platelets. Perhaps energy is required at specific time points during degranulation, and if these time-points are not achieved, then the degranulation machinery may become compromised and unable to proceed with degranulation. Currently, whether GLUT3 plays a role in sequential degranulation cannot be tested due to technological limitations.

Glucose metabolism for platelet functions following activation

Platelets may facilitate postactivation glucose uptake to fuel functions following, which occur after activation. Platelet functions following activation are not well understood, but activated platelets are known to facilitate clot retraction (26) and microparticle formation. Manipulation of postactivation metabolism may aid in understanding the functions that platelets facilitate following activation.

Clot retraction is a platelet-dependent mechanism, which occurs following activation. In vitro studies indicate that in the absence of glucose, clot retraction does not occur (26). Although the ability of GLUT3-KO platelets to undergo clot retraction was not assessed in vitro, in an in vivo model of deep vein thrombosis, clot formation was unaffected. Although in vivo deep vein thrombosis and clot retraction are not the same, these data suggest that postactivation glucose uptake may not be essential for clot maintenance in vivo.

Following activation, platelets can produce microparticles, which account for ~80% of microparticles in the circulation. Platelet-derived microparticles can play essential roles in rheumatoid arthritis disease progression as well other immune responses (27, 28). Additionally, microparticle formation is thought to require large amounts of energy for membrane-cytoskeleton adhesion and membrane remodeling. Therefore, it is plausible that postactivation glucose uptake aids in microparticle formation. This is accentuated by the observation that in vitro stimulation of platelets with collagen plus thrombin leads to opening of the mitochondrial permeability transition pore (MPTP), which results in mitochondrial depolarization, yet microparticles are

subsequently formed hours later. Because this stimulus abolishes mitochondrial respiration, glucose metabolism acts as the sole pathway for ATP production in this culture system. It is not clear whether the basal rate of glucose uptake can maintain energy levels for microparticle formation, because microparticle formation was not assessed in GLUT3-KO platelets. However, in a KBxN model of autoimmune inflammatory arthritis, which is dependent on microparticle formation, GLUT3-KO mice display decreased disease progression. Studies of GLUT3-KO platelets may aid in understanding the mechanism by which platelets generate microparticles.

Finally, although unlikely, postactivation glucose uptake could be an artifact of GLUT3 localization to α-granules. Recent studies have shown that under basal conditions GLUT3 facilitates glucose uptake into α-granules. Therefore, perhaps, translocation of α-granules into the plasma membrane, leading to the increased capacity for glucose uptake, is only due to a coincidental localization. We now know that GLUT1 and GLUT3 display redundant functions, and that they do not appear to operate at maximal levels under basal conditions. This finding was based on their ability to compensate in the absence of one another. It may thus be possible that activation-mediated increased glucose metabolism can occur independent of GLUT3 translocation, by regulation of machinery downstream of glucose transporters. In addition, platelet activation leads to increased consumption of ATP and activation of AMPK. This activation may lead to a compensatory increase in glucose metabolism to protect the energy state, with no bearing on cellular energy demands.

Although the contribution of postactivation glucose uptake does not appear to aid in degranulation, the functional consequence remains enigmatic. The contribution of postactivation glucose uptake to clot retraction cannot be ruled out; however, a role for postactivation glucose uptake in clot formation in vivo seems unlikely. The contribution of postactivation glucose uptake to microparticle formation provides a provocative mechanism by which microparticle formation may be regulated. Further investigation of these mechanisms may advance the understanding of postactivation platelet functions and why platelet activation leads to this

dramatic increase in glucose metabolism.

Glycogen

Glycogen is composed of hundreds to hundreds of thousands of glucose molecules linked together to form heterogeneous polymers (29). Glycogen maintenance is a dynamic process: glycogen synthase facilitates the polymerization of glucose to glycogen, while glycogen phosphorylase converts glycogen to glucose-1-phosphate for subsequent metabolism (29). Platelets contain large quantities of glycogen, which are visible by transmission electron microscopy at a density similar to skeletal muscle (6). This abundance is emphasized by the fact that in the absence of glucose and in the presence of the mitochondrial electron transport chain inhibitor cyanide, glycogen stores can maintain platelet ATP levels for up to 40 minutes (30). To date, however, the significance of glycogen utilization to platelet function remains unknown.

It is not clear whether platelet glycogen stores are generated *de novo* once in the circulation or if megakaryocytes package glycogen into nascent platelets prior to platelet budding. Megakaryocytes contain large concentrations of glycogen that fluctuate throughout development, with the largest concentration present just prior to pro-platelet formation (31, 32). Following platelet formation, megakaryocyte glycogen is absent (32); however, it is unclear whether megakaryocytes consume the glycogen stores for energy to generate platelets or if the glycogen is packaged into the nascent platelets.

Platelet glycogen content can be maintained while in the circulation. Incubations of washed platelets with C^{14}-glucose leads to a significant accumulation of C^{14}-labeled glycogen (30), indicating glycogen can be synthesized in platelets. Additionally, platelets also incorporate C^{14}-citrate and C^{14}-pyruvate into glycogen, indicating the presence of glyconeogenesis, although the rate of accumulation was quite small (30). Moreover, murine platelets lacking GLUT3 display markedly reduced glycogen; however, megakaryocytes from these mice appear to have normal glycogen content. This finding indicates that impaired glucose uptake in the circulating

platelet may lead to increased glycogen utilization, or decreased glycogen synthesis. These data demonstrate that platelets are capable of utilizing multiple substrates to maintain glycogen levels after budding from megakaryocytes.

Thrombin-mediated platelet activation leads to the utilization of glycogen (6) through GSK and AKT signaling (33), leading to inhibition of glycogen synthase and decreased glycogen synthesis. Also, platelets express neuronal-, muscle-, and liver-type glycogen phosphorylase, which facilitates glycogen breakdown (6) following platelet activation. Thus, activation-mediated glycogen utilization may play an important role in energy utilization for platelet activation.

Recent studies investigating the contribution of glycogen content to α-granule release indicate that glycogen-mediated metabolism contributes to platelet degranulation. This was demonstrated in platelets depleted of their glycogen stores following incubation in glucose-free media. Platelets from the same animal, but containing less glycogen, display a modest decrease in CD62p surface translocation, a maker of α-granule degranulation (Chapter 2). These data demonstrate that glycogen utilization at the time of activation is not just correlative, but aids in degranulation.

Pentose phosphate pathway

Platelets are capable of metabolizing glucose through the PPP, which is directly linked to NADPH biosynthesis and GSH cycling (34). Platelets treated with *t*-butyl hydroperoxide display a 50-fold increase in PPP flux, with no alteration in glucose oxidation, indicating that PPP flux can be regulated by ROS, presumably through the consumption of GSH (34). Platelet activation by most agonists leads to increased COX-1 activity, which can convert arachidonic acid (AA) to the potent platelet agonist thromboxane A_2, amplifying platelet activation. In fact, AA is not only metabolized through COX-1, but also by lipoxygenase (LOX5 and LOX12), which can lead to the formation of multiple HETEs and leukotrienes, which play important roles in platelet activation, immune response, and inflammation (35). Interestingly, in vitro incubations of

platelets with AA in the presence of COX-1 inhibitors (to prevent activation) increase PPP flux ~10-fold. In the presence of the lipoxygenase inhibitor 15-HETE, this AA-mediated increase is partially reversed, indicating that AA metabolism is associated with increased PPP flux (34). Furthermore, by depleting GSH levels, it was shown that glutathione peroxidase is likely responsible for the reduction of the LOX-12 product 12-HpETE to 12-HETE. Because GSH levels mediated this conversion, it is probable that the relationship between PPP flux and AA metabolism is regulated through the utilization of GSH. Additionally, in the absence of glucose, the formation of α-HEPA (the result of an alternative metabolic pathway to LOX-mediated formation of 12-HETE) was increased. When 1mM glucose was added back to the media, formation of these metabolites was greatly reduced (34). NADPH and GSH have been shown to be essential regulators of platelet function (36-39). Therefore, modulation of PPP flux may be a direct link between glucose metabolism, ROS production and platelet activation. Future studies investigating the role of PPP-mediated glucose metabolism and platelet function are needed to better understand this relationship.

Polyol pathway

Platelets can metabolize glucose to sorbitol and fructose through the polyol pathway (40). This process requires aldose reductase to convert glucose to sorbitol in an NADPH-dependent manner. Sorbitol can then be metabolized to fructose through sorbitol dehydrogenase, which reduces NAD+ to NADH. Finally, fructokinase can phosphorylate fructose-yielding fructose-6-phosphate, which can then proceed through glycolysis (41). Under basal conditions, the significance of this pathway is unknown; however, platelets incubated in high-glucose (25mM) media display increased aldose reductase activity (40) and, when treated with collagen, exhibit increased aggregation and P-selectin surface translocation, which is reversible in the presence of aldose reductase inhibitors (42). This increased activation may be due to increased ROS, which can be formed due to the increased utilization of NADPH by aldose reductase, leading to

decreased GSH-GSSG cycling, increased ROS, and possibly increased platelet activation (38). In addition, aldose reductase transgenic mice with T1DM display increased in vivo thrombosis and in vitro aggregation. These increases were associated with increased intracellular ROS (42) and decreased in vitro by administration of aldose reductase inhibitors. Whether aldose reductase activity is dysregulated in disease states, and whether glucose flux through this pathway can alter platelet function and NADPH levels still remain to be determined.

Metabolomics analysis of GLUT3-deficient platelets under basal conditions revealed a marked reduction in the polyol pathway intermediates sorbitol and fructose. Although under basal conditions GLUT3-KO platelets have ~20% reduced glucose uptake, this decrease in polyol pathway intermediates in GLUT3 platelets was not accompanied by large changes in other glycolytic pathways. These data indicate that GLUT3 selectively facilitates polyol pathway flux, although the mechanism by which this relationship is facilitated is unknown.

GLUT3-mediated polyol pathway metabolism may be facilitated through three possible mechanisms. It is possible that glucose entry into the polyol pathway may be regulated by aldose reductase, which may have a lower affinity for glucose than do glycolytic or PPP enzymes. Because the exact contribution of polyol pathway flux is unknown and its regulation appears to be dependent on substrate availability, perhaps flux through this pathway is more sensitive to changes in glucose availability. Additionally, polyol pathway metabolism may occur in the α-granules, and therefore require GLUT3-mediated entry into the α-granule. Although proteomic analysis of α-granule proteins and platelet releasate did not indicate the presence of aldose reductase or sorbitol dehydrogenase, this lack of detection may have occurred due to technical limitations. Future studies will be required to better understand the contribution of GLUT3 to polyol pathway metabolism and why this pathway may be more responsive to glucose abundance.

Platelet function in the absence of glucose metabolism

The generation of DKO mice allowed us for the first time to determine the contribution of glucose metabolism to platelet function in vivo. DKO platelets have completely abolished glucose uptake and dramatically decreased glycolytic rate. Evaluation of these mice demonstrated that glucose metabolism is essential for platelet survival and function in vitro and in vivo.

Glucose metabolism is essential to maintain platelet homeostasis

DKO mice displayed ~30% reduction in platelet counts. This decrease in platelet counts was attributed to a reduction in circulating-platelet half-life. The mechanisms regulating circulating-platelet half-life are not well understood, but it is known that platelet apoptosis generates an "eat me" signal to phagocytic cells, which facilitates clearance of platelets (43). Interestingly, in the absence of glucose metabolism, platelets undergo apoptosis, which can be partially inhibited in vitro when substrates for mitochondrial metabolism, pyruvate and glutamate, are present. Even in the presence of mitochondrial substrates, which would be found in the plasma, DKO platelets displayed a marked increase in apoptosis. The idea that metabolism can regulate life-span is not new (44); however, these studies are the first ones to demonstrate that a reduction in platelet glucose metabolism can lead to decreased circulating half-life. Studies of human platelets, where platelet density was used to distinguish platelet age, indicated that dense (young) platelets contained larger quantities of glycogen, and had increased respiration and glycolysis rates compared to light (old) platelets (44-46). These studies demonstrate that metabolism can regulate platelet apoptosis and circulating lifespan.

Platelet dysfunction in diabetes

Patients with T1DM and T2DM have increased thrombosis and microvascular and macrovascular disease (47, 48). These pathologies arise from multiple facets, including alteration in the complement system, endothelial dysfunction (47), systemic inflammation (49), and

dysfunctional platelets (50). Platelet dysfunction in diabetes is somewhat unclear due to lack of reproducibility in the multiple studies in the literature. This variability in the literature may be due to a divergent response of platelets to diabetes or perhaps due to alterations in subcellular energy production. Two major hallmarks of diabetes are increased circulating glucose and increased systemic inflammation (49). Systemic inflammation likely plays a major role in altering platelet function in diabetes, because platelets express TLR4 (51). Here we will discuss the contribution of glucose metabolism to platelet dysfunction in T1DM and T2DM.

Patients with T1DM (52) and T2DM (53) display increased urinary secretion of 11-dehydro-thromboxane B_2, an in vivo marker of thromboxane A_2 production, which itself is a marker of platelet activation. In addition, whole blood isolated from patients with T1DM contains increased platelet-monocyte aggregates, used as a marker of increased in vivo platelet activation and degranulation (54). Patients with T2DM subjected to acute hyperglycemia using a hyperglycemic clamp display decreased bleeding time, increased sheer stress-induced platelet activation, and increased 11-dehydro-thromboxane B_2 urinary secretion (55). These studies largely represent the current knowledge of in vivo platelet dysfunction in diabetes. Further, ex vivo studies have indicated that platelets from diabetic patients are inherently dysfunctional. Platelets isolated from diabetic subjects demonstrate increased "stickiness" when incubated in a glass bulb (56), increased binding to fibrinogen (57), increased sCD40L release (54, 58), and increased aggregation in response to ADP (57) and collagen (42). In mice, there is increased GPIIbIIIa activation in response to PAR4 peptide (59). To date, only one study has investigated the effect of diabetes on glucose metabolism in platelets, and this was done in streptozotocin (STZ)-treated rats, which are a model of T1DM. Interestingly, these rats had increased ex vivo platelet aggregation and accumulation of the glycolysis intermediates: glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-bisphosphate, 3-phosphoglycerate, 2-phophoglycerate, phosphoenolpyruvate, and pyruvate (9), indicating that glucose metabolism may be altered in the diabetic platelet. Because platelet activation is so tightly coupled to ATP energy homeostasis,

alterations in glycolysis may lead to more excitable platelets. Future studies are required to investigate this relationship.

Conclusions

Many questions remain regarding the contribution of glucose metabolism to platelet function. The extent to which diabetes or metabolic syndrome alters glucose metabolism in human platelets needs to be determined. Additionally, basic questions remain as to why platelets increase glucose metabolism following degranulation. Understanding these postactivation consequences of metabolism may aid in our understanding of thrombus clearance, wound healing, and other unknown platelet functions. In addition, the contribution of glucose metabolism to platelet ROS formation and lipoxygenase activity could represent interesting mechanisms by which the extracellular milieu may regulate platelet function and thrombosis. Moreover, understanding if α-granule glucose flux is altered in disease states may be important for the generation of biomarkers. Generation of GLUT1- and GLUT3-deficient platelets has allowed these questions and others to be tested and will aid in a better understanding of how the fundamental process of glucose metabolism can lead to alterations in platelet function in vivo.

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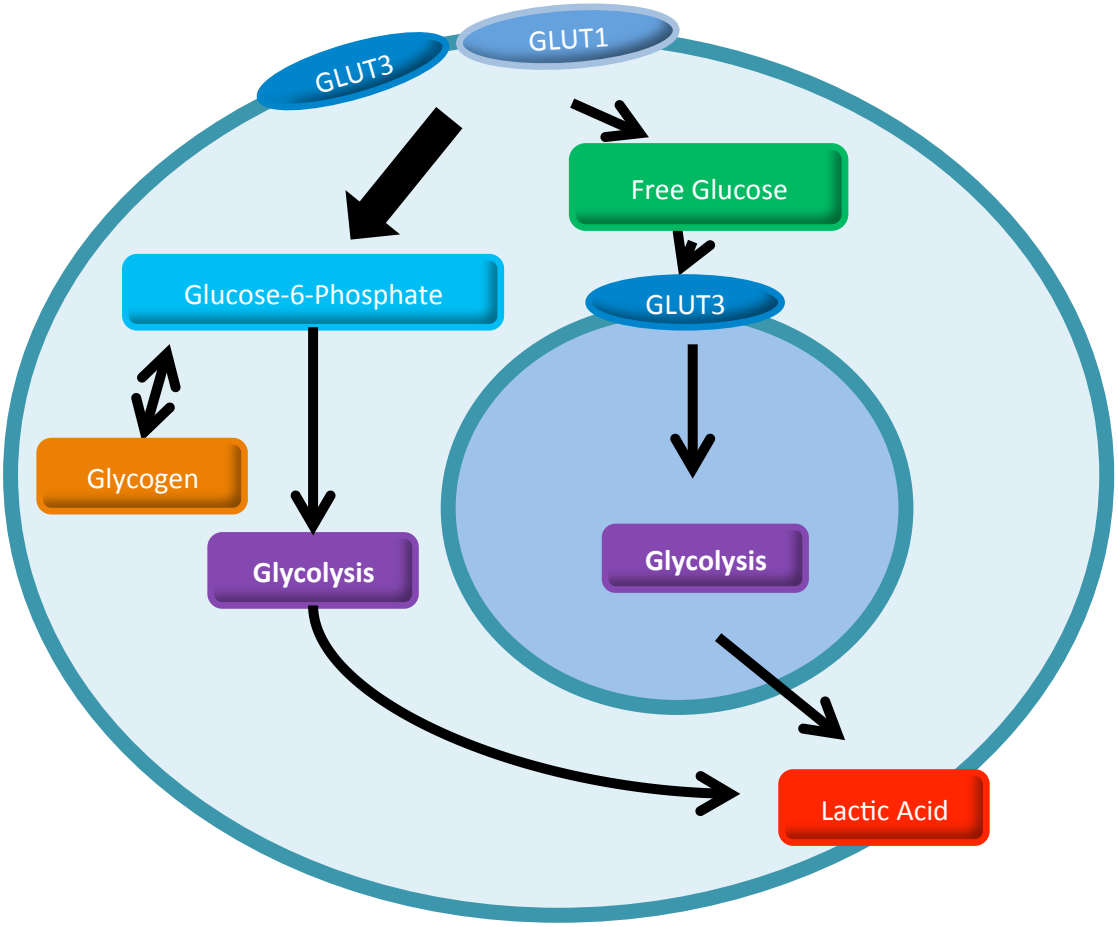

Figure 4.1 Platelets undergo intragranular glycolysis.

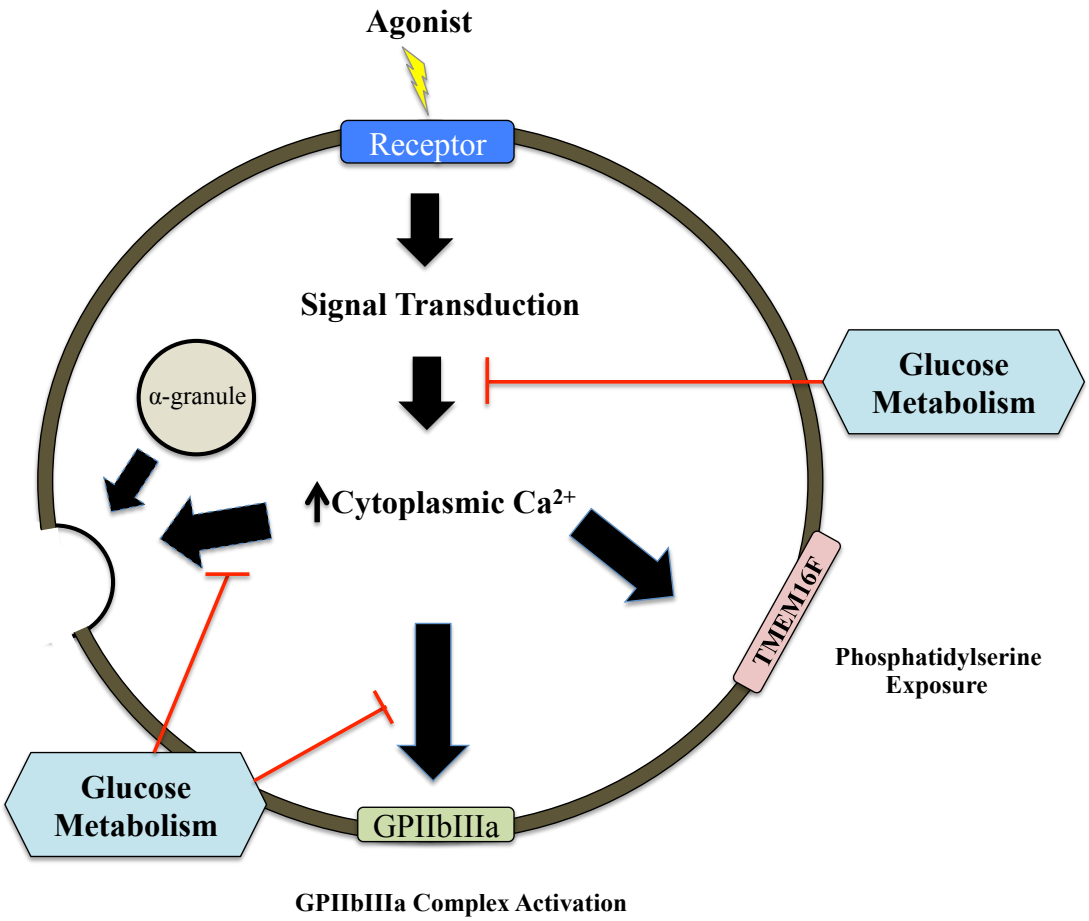

Figure 4.2 Glucose metabolism regulates multiple aspects of platelet activation.